HOMOLOGOUS PAIRING OF DNA MOLECULES PROMOTED BY REC1 PROTEIN OF *USTILAGO MAYDIS*

By

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To my parents, Emil and Catherine, and my wife, Jennifer

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KEY TO ABBREVIATIONS

AMPPNP	adenylyl-imidodiphosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CTP	cytosine-5'-triphosphate
d	dalton
dCTP	deoxycytosine-5'-triphosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
Kd	1,000 daltons
PMSF	phenylmethylsulfonylfluoride
poly(dC)	polydeoxycytidylic acid
poly(dG)	polydeoxyguanylic acid
RF I	form I, superhelical DNA
RF II	form II, nicked circular DNA
RF III	form III, linear DNA
RF IV	form IV, relaxed closed circular DNA
RNA	ribonucleic acid
rpm	revolutions per minute

SDS	sodium dodecyl sulfate
SSC	standard saline citrate (0.15 M NaCl, 15 mM sodium citrate)
u	units
Z-DNA	left-handed helical double stranded DNA

Abstract of Dissertation Presented to the Graduate School
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HOMOLOGOUS PAIRING OF DNA MOLECULES PROMOTED BY REC1 PROTEIN OF USTILAGO MAYDIS

By

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An enzyme was isolated from mitotic cells of the fungus Ustilago maydis based on its ability to reanneal complementary strands of DNA. The purified protein of molecular weight 70,000 daltons catalyzes the ATP-dependent pairing of a wide range of DNA substrates including the uptake of single stranded DNA by a homologous superhelical duplex. In addition, circular single strands of DNA are paired with homologous linear duplex molecules in a reaction occurring in two distinct and experimentally separable stages. In the first phase, synapsis, the enzyme aligns the DNA molecules and brings them into homologous register. The second phase, strand exchange, extends the length of the heteroduplex joint in a protein-directed polar fashion. Synapsis requires only the presence of ATP, while strand exchange utilizes the energy of ATP hydrolysis. Furthermore, synapsis entails the formation of a ternary complex composed of the two DNA molecules and rec1 protein. When the synaptic complexes are formed in the absence of a

free end, the topological constraint is relieved by the development of stretches of left-handed DNA. A complex series of steps leading to synapsis comprises a third phase of the strand transfer reaction. During the presynapsis phase, ADP and single stranded DNA regulate the synaptic pairing activity of rec1 protein. Mutations in the rec1 gene of Ustilago lack this ATP-dependent pairing activity. Because it is the first enzyme isolated in eukaryotes having such recombinational properties and because the rec1 mutation from Ustilago is deficient in some forms of recombination, the enzyme has been called rec1 protein.

CHAPTER ONE
INTRODUCTION AND DEVELOPMENT OF ASSAY SYSTEMS

Genetic recombination is a process by which linkage relationships among genes are altered by a breakage and reunion of homologous chromosomes (Meselson, 1967). The rejoining mechanism involves the pairing of complementary sequences of single strands of DNA contributed by different parent duplexes. The structure created by this molecular splicing is often referred to as a heteroduplex joint. On a molecular basis, a variety of models have been proposed to account for the formation of the heteroduplex joint (Holliday, 1964; Whitehouse, 1973; Meselson and Radding, 1975). It is currently believed that recombination begins with the transfer of a single strand of DNA from one duplex molecule to another. Subsequently, this strand pairs with its complement and displaces its homolog. The structure, thus created, is commonly referred to as a D-loop.

The biochemical nature of genetic recombination has only recently come into focus. The remarkable E. coli protein recA, indispensible for reciprocal recombination, has been shown to align DNA molecules into homologous register and promote the formation of heteroduplex molecules (McEntee and Epstein, 1977; Shibata et al., 1979; Cunningham et al., 1980; Kahn et al., 1981; Cox and Lehman, 1981; Gonda and Radding, 1983). The pairing of homologous DNA molecules is a crucial event in the overall process of reciprocal recombination.

The bacteriophage λ, can carry out homologous recombination in the absence of functional recA protein relying instead on its own red system (Signer and Weil, 1968). This two gene system was shown to code for an exonuclease with a 5' to 3' specificity (Little, 1967), and a protein, known as β protein, whose function was unknown. An attractive possible activity for β protein would be that the pairing of DNA strands, a function required for the initiation of recombinational processes.

To study this possibility an assay was designed in which [^{3}H]-labeled P22 single strands of DNA become resistant to the single strand specific nuclease, S-1 (Weinstock et al., 1979). The development of such resistance could be a reflection of the strands assuming a duplex or paired DNA form. P22 was chosen as a substrate because of its complex nature and slow natural renaturation. To avoid misrepresenting results, DNA from the single stranded circular bacteriophage ϕX was digested by S-1 nuclease after β protein treatment. In this way, the resistance to S-1 nuclease due to simple protein-DNA binding could be accounted for.

Purification of β Protein

The procedure for purification of β protein outlined by Radding et al. (1971) was followed with slight modifications (see Appendix A). In general, the λ exonuclease and β protein co-chromatograph throughout the purification procedure. They were fractionated and separated by chromatography on phosphocellulose which produced the activity profile illustrated in Figure 1-1. SDS gel electrophoresis (Fig. 1-2) of two peaks (I and II) depicted in Figure 1-1 produces a pattern consistent with that reported by Radding et al. (1971). β protein was identified

Fig. 1-1. Chromatography of red gene products.

Fraction IV containing partially purified exonuclease and β protein was chromatographed on phosphocellulose as described in Appendix D. The column was run at 30 ml/hour and fractions of 5 ml each were collected and monitored for exonuclease (○), reannealing activity (●), and A_{280} (△).

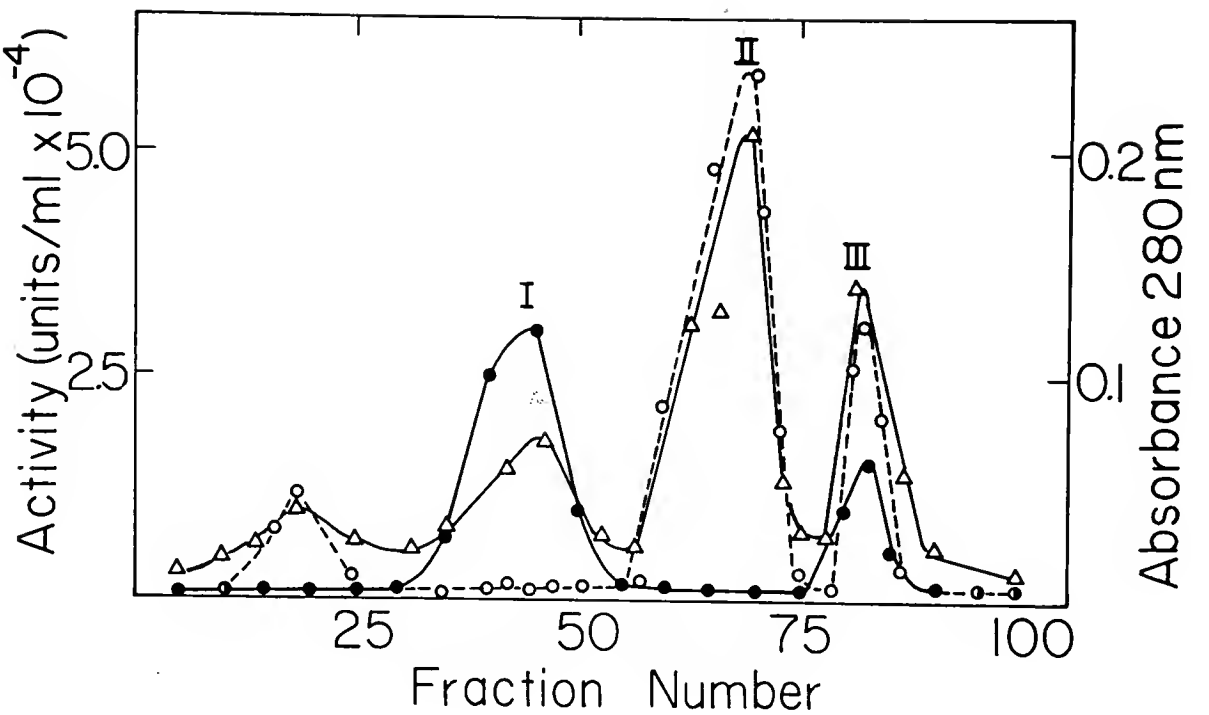
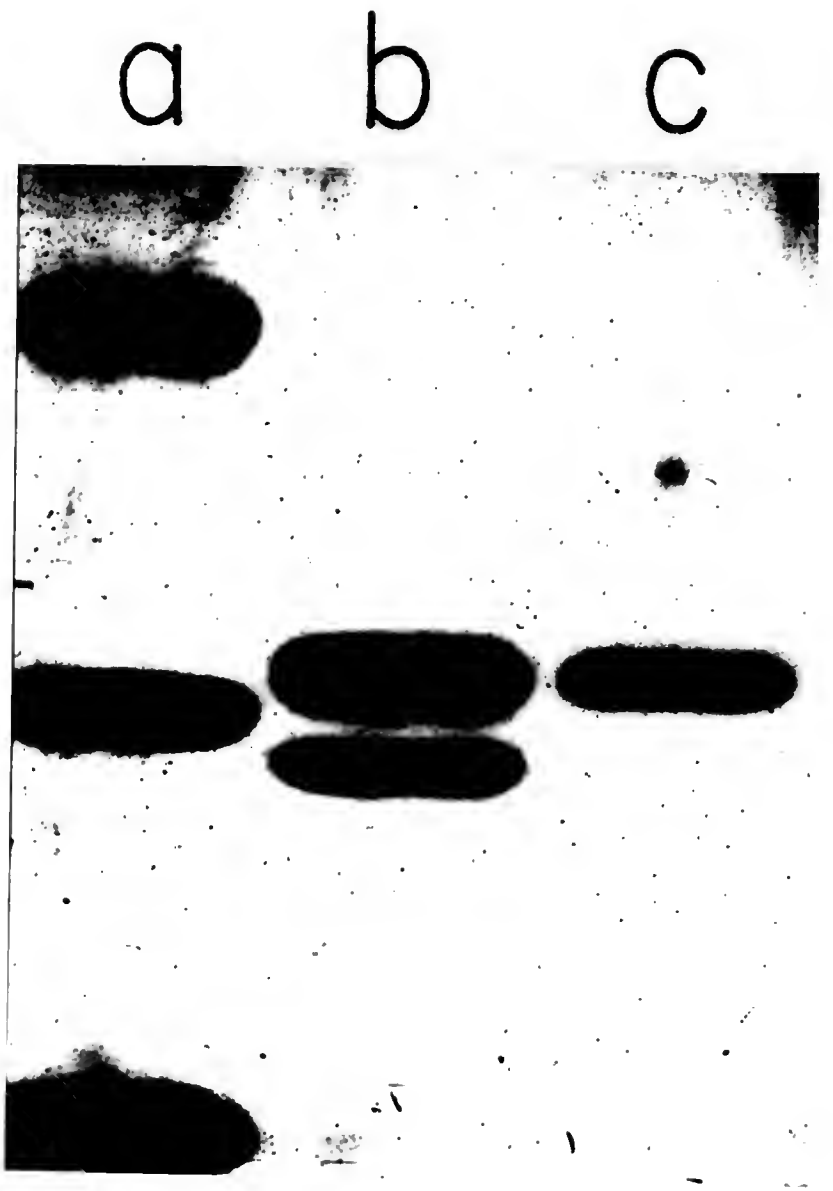

Activity (units/ml x 10^{-4})
5.0
2.5
I
II
III
0.2
0.1
Absorbance 280nm
25
50
75
100
Fraction Number

Fig. 1-2. SDS gel electrophoresis of purified β protein.
Samples containing 10 to 20 μg of protein from Peaks I and III shown in Figure 1-1 were subjected to SDS gel electrophoresis as described in Appendix F. Molecular weight standards include ovalbumin (43,000) α-chymotrypsinogen (25,700) and lysozyme (14,300).
a) Standards, b) Peak III, c) Peak I.


a
b
c

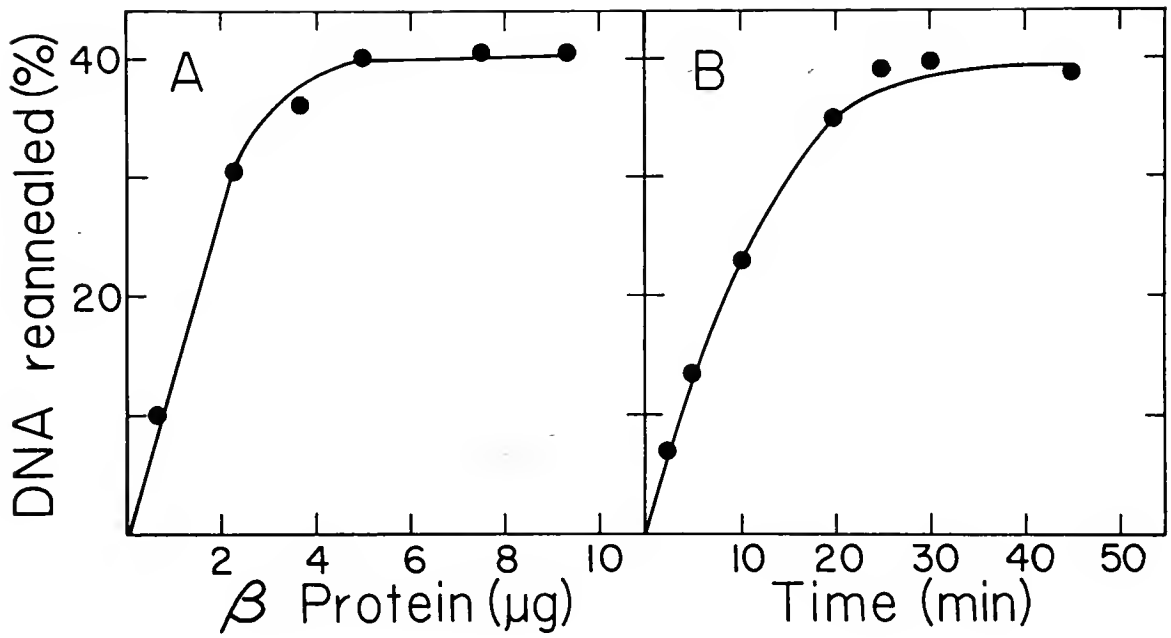
by its characteristic molecular weight (28,000 daltons) and found to be present in Peak I (lane c). Peak II contained the λ exonuclease. Peak III in the phosphocellulose profile contained the α exonuclease β protein complex as described by Radding et al. (1971). Both this complex and the α exonuclease alone contained high levels of nuclease activity (65,000 units/mg).

β Protein Promotes DNA Reannealing

Fractions from Peaks I, II, and III were assayed for their capacity to reanneal complementary strands of DNA as determined by the S-1 nuclease assay. Peak I and III, but not II, contained high levels of this activity. Neither peak catalyzed the formation of S-1 resistant DNA molecules when incubated with single strand circular DNA. One unit of reannealing was defined as the amount of β protein capable of reannealing 10 picomoles of P22 single stranded DNA in 30 minutes at 37°C.

The reaction requirements included a phosphate buffer near pH 6.0 and a divalent cation. Magnesium-chloride ($MgCl_2$) was the most effective metal used although calcium chloride ($CaCl_2$) produced approximately 30% reannealing activity. The fraction containing the most homogeneous preparation of β protein was used in the characterization studies. The specific activity of this fraction was approximately 1.26×10^{-5} units/mg (Reannealing). The titration of β protein as a function of its reannealing activity is depicted in Figure 1-3B. The curve rises sharply and finally plateaus. The reaction is also time dependent reaching a maximum level at 20 minutes. The amount of β protein used to determine the time course of reannealing was 5 μg. At saturation, there was approximately 1 molecule of β protein/65

Fig. 1-3. Requirements of Reannealing Reaction.
Kinetics of reannealing. A. Reaction mixtures prepared as described in Appendix F containing the indicated amounts of β protein were incubated at 37°C for 30 minutes. B. Individual reaction mixtures were prepared and incubated with 5 μg β protein. At the indicated time each sample was processed as described in Appendix E.


DNA reannealed (%)
40
20
A
B
2
4
6
8
10
β Protein (μg)
10
20
30
40
50
Time (min)

nucleotides of DNA (Kmiec and Holloman, 1981). The maximum amount of single stranded P22 DNA becoming S-1 nuclease resistant was only 44% of input. This somewhat low value may be attributed to the fact that P22 DNA may form large aggregates in solution incapable of becoming reannealed. When single stranded P22 DNA was thermally reannealed at 65°C for 3 hours, approximately 55% of input became resistant to S-1 nuclease digestion.

To explore the possibility that both gene products of the red system may work in a coordinated fashion and to confirm that the DNA was actually becoming reannealed, single stranded [^{3}H]-labeled, P22 DNA was treated with β protein for 30 minutes at 37°C. After incubation, the DNA was digested with α exonuclease, whose specificity is for duplex DNA. Figure 1-4 illustrates that the P22 DNA became increasingly sensitive to α exonuclease digestion as a function of exposure time to β protein.

Reannealing Activity in Recombination-Deficient Mutants

A variety of phage λ recombination mutants are available and provide the opportunity to directly implicate β protein as an enzyme which catalyzes the reannealing of DNA molecules. Cells in miniculture (2 liter volumes) were processed through the phosphocellulose step in the purification scheme of β protein. As shown in Table 1-1, the induced red$^+$ lysogen contained high levels of exonuclease and reannealing activity. However, mutations in either the exonuclease gene (λ red X314) or β protein genes (B113) (Radding et al., 1971) showed significant loss of nuclease or reannealing activity respectively.

Fig. 1-4. Activity of λ exonuclease on denatured DNA treated with β protein.

A reaction mixture (300 µl) containing 10 mM potassium phosphate pH 6.0, 10 mM $MgCl_2$ 21 nmol denatured P22 [^{3}H]DNA and 45 µg β protein was incubated at 37°C. At the indicated times, aliquots (0.05 ml) were removed, mixed with 0.25 ml of 50 mM glycine·KOH, pH 9.5, 10 mM $MgCl_2$, 6.5 units of λ exonuclease (α protein) and incubated an additional 30 minutes. DNA rendered acid soluble was determined as described in Appendix E.

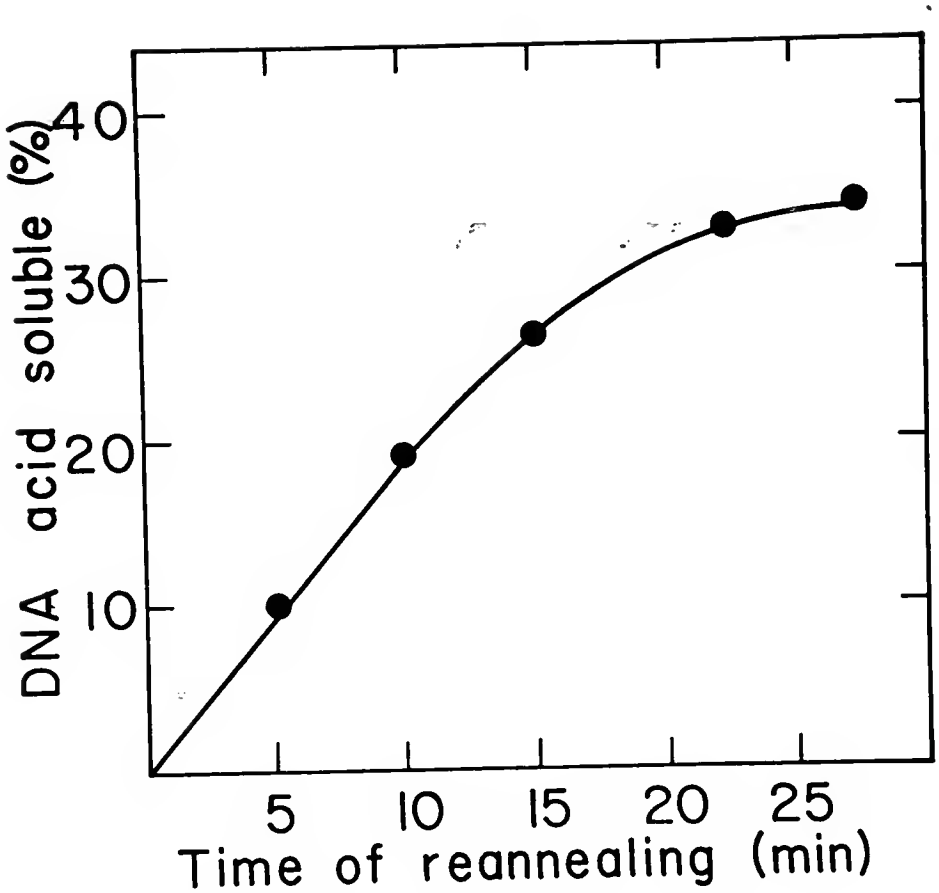

DNA acid soluble (%)
40
30
20
10
5
10
15
20
25
Time of reannealing (min)

TABLE 1-1

Levels of β Protein and Exonuclease in Red Mutants Lysogens

Lysogen	Exonuclease units/mg	Reannealing units/mg x 10^{-3}
AB2463 (λred$^+$)	900	126
AB2463 (λred$^+$) uninduced	2.0	< 2.0
AB2463 (λred X314)	0.8	186
AB2463 (λred B113)	830	< 2.0

Cell extracts were prepared from lysogens as described in Appendix D and chromatographed on DEAE-cellulose and phosphocellulose. Specific activities for exonuclease were determined in crude lysates. Specific activities for β protein promoted reannealing were determined after phosphocellulose chromatography.

Discussion

β protein from phage λ was found to catalyze the reannealing of complementary single strands of DNA in an assay which measured increased resistance to S-1 nuclease. The enzyme was also found to be capable of cooperative enzymatic activity with another gene product from the same genetic loci. β protein's enzymatic activity was verified by mutant studies in which recombination deficient phage λ strains lacked homologous pairing activity (due to the loss of functional β protein). These results also validate the use of the S-1 nuclease assay in probing extracts of cells for DNA pairing activity.

The results presented here provide a function for β protein in generalized recombination events in phage λ. Although the recA gene product could substitute for β protein within cells undergoing recombination, the reaction mechanisms of the two proteins are quite different. For example, recA protein requires adenosine triphosphate (ATP) for renaturation reactions, while β protein does not. This difference may ensure that specific DNA metabolic processes are able to occur independent of environmental limitations. Furthermore, the red (phage λ) and rec (*E. coli*) systems of recombination are functionally unrelated to a large extent (Shulman *et al.*, 1970). Other proteins, such as the gene 32 protein from T4 phage, have also been found to catalyze the reannealing of single strands of DNA (Alberts and Frey, 1970). Mutations in this gene product are defective in T4 recombination.

CHAPTER TWO
USTILAGO MAYDIS CONTAINS AN ENZYME WHICH CATALYTICALLY PAIRS HOMOLOGOUS DNA MOLECULES

Yeast and fungal cells have served as model systems for the study of genetic recombination in eukaryotes. Since both these organisms can be studied in the haploid and diploid states, results gained from such analysis develop our appreciation for the complexities of recombination in higher organisms. These studies have revealed a number of different types of recombination events. For example, haploid mitotic cells from yeast can undergo both intrachromosomal recombination and sister chromatid exchange (Jackson and Fink, 1981). Gene conversion and reciprocal recombination occurring in fungi are often closely associated in meiosis (Case and Giles, 1969). Lesions in DNA molecules have been found to induce recombination events in mitotic cells, probably by inducing certain recombination-repair pathways (Fabre and Roman, 1977). Such an association between DNA breaks and meiosis has not been established. Resnick and Martin (1976) showed that double strand breaks may be initiation sites for recombination and recently Szostak *et al*. (1983) showed that double strand breaks are recombinogenic.

Mitotic recombination occurs prior to DNA replication with the creation of symmetric Holliday structures initiated by the asymmetric transfer of a single strand of DNA into a sister duplex (Meselson and Radding, 1975). Recently, a mutation in yeast cells has been

identified that diminishes meiotic recombination, but enhances mitotic recombination. Furthermore, it provides strong genetic evidence for the existence of an asymmetric exchange event (Bruschi and Esposito, 1983).

This asymmetric exchange event of DNA strands produces a structure known as a heteroduplex joint, a molecular splice in which two homologous DNA strands contributed by different parent duplexes are paired via hydrogen bonds. How the formation of the heteroduplex joint occurs has been a puzzling question. The discovery and purification of the recA gene product from Escherichia coli has provided insight into a possible mechanism for the formation of this crossover junction (Cunningham et al., 1981). This enzyme of molecular weight 40,000 daltons pairs a wide range of homologous DNA molecules using the energy generated by the hydrolysis of ATP (Shibata et al., 1979; Weinstock et al., 1979; DasGupta et al., 1980; Cox and Lehman, 1981; West et al., 1981). The underlying feature of all recA protein-catalyzed reactions is its ATP-dependent reannealing of complementary DNA strands. The wide range of structures created from this activity resemble structures highlighted in the recombination models of Holliday (1964), Meselson and Radding (1975), Wilson (1979) and Szostak et al. (1983).

Biochemical studies of heteroduplex formation in eukaryotes have lagged far behind those in prokaryotes. However, the genetic and physical analyses of eukaryotic recombination amassed by numerous laboratories provide a solid basis for such enzymological investigations. Using the experimental approach devised in studying the recA protein, but realizing the increased complexity of recombination

mechanisms in eukaryotes, extracts from the fungus Ustilago maydis were examined for the presence of a DNA pairing activity.

Isolation and Purification of a Pairing Protein

As previously established, a convenient way to measure the reannealing of complementary single stranded DNA is by using S-1 nuclease to digest any unpaired DNA (Weinstock et al., 1979; Kmiec and Holloman, 1981). There are a number of limitations in using this particular assay. First, DNA binding proteins could protect the DNA against S-1 nuclease digestion. Second, a variety of other cellular components, histones and polyamines (Cox and Lehman, 1981) promote the noncatalytic renaturation of DNA contributing significantly to higher assay backgrounds. As a control ϕX single strand circular DNA, incapable of being reannealed itself, was used to measure the blocking of S-1 nuclease digestion. In addition, only reannealing activity which was dependent on the presence, and subsequently the hydrolysis of ATP, was characterized further. Reaction mixtures generally contained recently denatured P22 DNA and ATP.

The cell extract resuspended in a buffer containing one molar KCl was initially fractionated by polyethylene glycol phase partition to remove DNA, polyamines, and DNA associated proteins such as histones. A small, but detectable amount of ATP-dependent reannealing activty was observed in this initial purification step. Isolation of the pairing protein continued by ion exchange chromatography on DEAE-cellulose and phosphocellulose. Activity was detected by elution with salt gradients on both columns. The anion exchanger, DEAE, was effective in removing much of the remaining nonspecific DNA binding activities. Phospho-cellulose chromatography proved to be an important purification step in

that it concentrated the ATP-dependent reannealing activity which eluted at a NaCl concentration of 0.35 molar. The main peak of activity co-eluted with a DNA-dependent ATPase activity. The purification scheme and phosphocellulose column profile of activity are depicted in Table 2-1 and Figure 2-1.

By using SDS polyacrylamide gel electrophoresis (PAGE) the protein responsible for the DNA pairing and ATPase activities was tentatively identified. A protein band with molecular weight 70,000 daltons was enhanced after chromatography on phosphocellulose, corresponding to the increased level of ATP-dependent reannealing (Figure 2-2, lane e). To remove a contaminating endonuclease activity, molecular sieve chromatography was employed. From this final purification step, the DNA-dependent ATPase and reannealng activity co-eluted with a molecular weight of 70,000 daltons. This observation was verified by electrophoresing the active fraction in an SDS PAGE system, again producing a 70,000 dalton protein (lane e) (see Appendix C).

DNA-Dependent ATPase Activity

The observation that a DNA dependent ATPase activity coeluted with the DNA reannealing activity proved to be important. Because this ATPase activity can be assayed conveniently, it provided supporting data throughout the purification procedure for identifying the appropriate pairing activity. Throughout the course of subsequent protein preparations, both assays were used simultaneously.

A distinctive characteristic of this ATPase was its dependence on the presence of single stranded DNA for high activity. No form of duplex DNA supported appreciable levels of activity when the most

TABLE 2-1

Purification of Rec1 Protein

Fraction	Volume (ml)	Protein (mg/ml)	ATPase	Reann. (u/ml)	Specific Activity ATPase	Specific Activity Reann.
Crude Extract	180	3.3	420		127.27	
P.E.G.	200	1.2	548		456.66	
DEAE-Cellulose	75	.6	822		1370.0	
Phosphoso-cellulose	25	.018	672	720	37,333	40,000
Agarose A 1.5 M	10	.006	914	390	152,333	60,000

Fig. 2-1. Co-chromatography of reannealing and ATPase activity.
A phosphocellulose column (1 x 12 cm) was loaded, washed and eluted as described in Appendix F. The flow rate was maintained at 30 ml/hour and fractions of 2 ml were collected. (○) Reannealing. (●) ATPase.

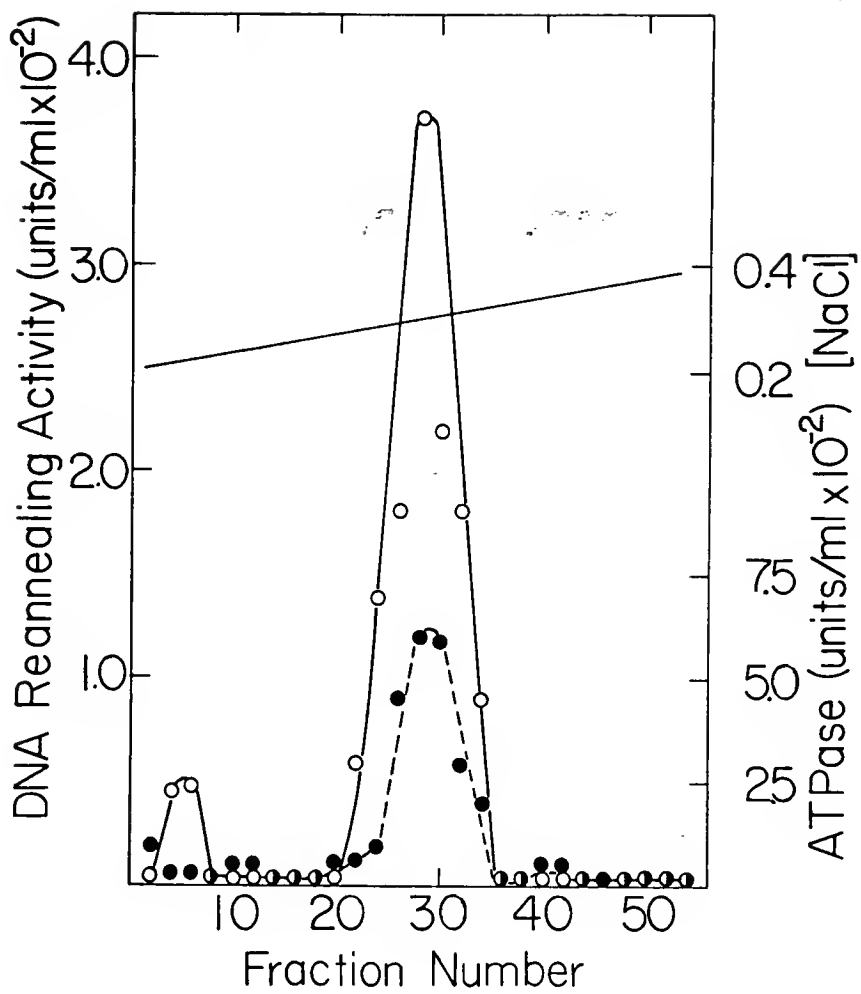
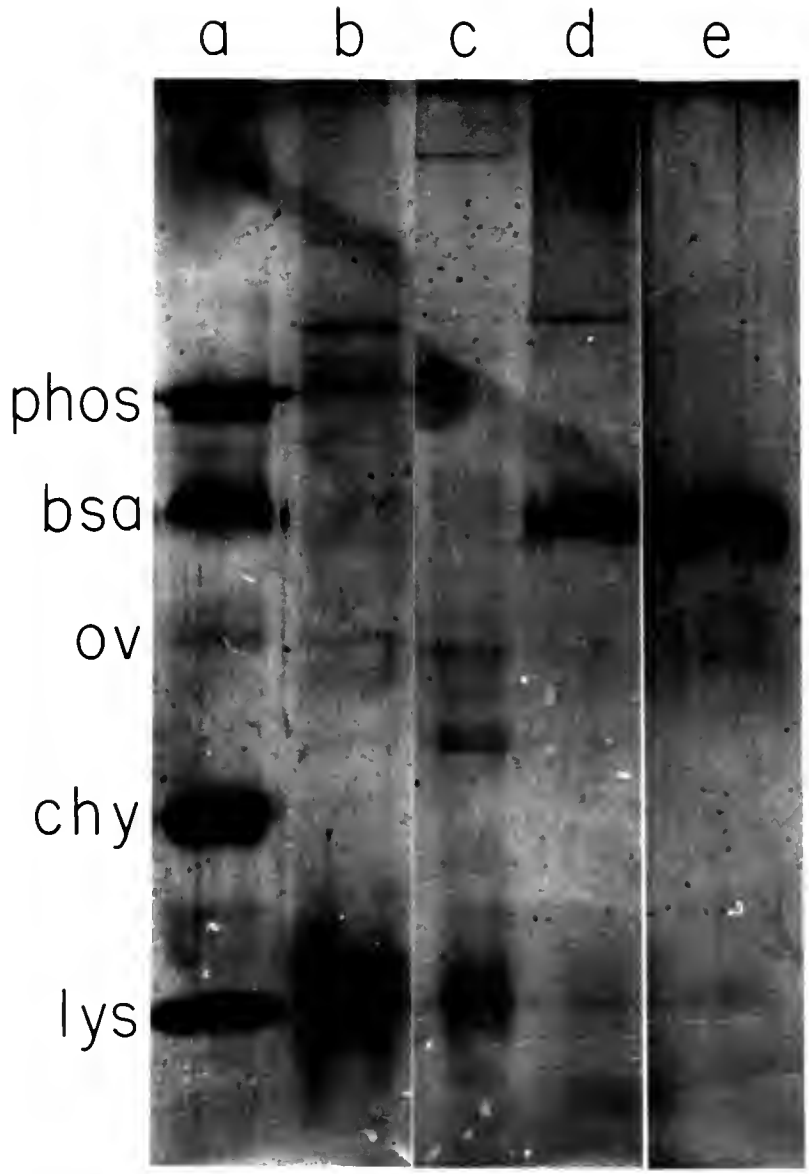

DNA Reannealing Activity (units/ml x 10⁻²)
4.0
3.0
2.0
1.0
10
20
30
40
50
Fraction Number
0.4
0.2
[NaCl]
7.5
5.0
2.5
ATPase (units/ml x 10⁻²)

Fig. 2-2. Purification of the Ustilago pairing protein.

Electrophoresis in a polyacrylamide gel containing SDS was performed as described in Appendix F. Channel a, standards for estimation of molecular weight include, from top to bottom, phosphorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), soybean trypsin inhibitor (Mr 21,000), channel b, polyethylene glycol Fraction II, 24 µg; channel c, DEAE-cellulose Fraction III, 18 µg; channel d, phosphocellulose Fraction IV, 0.8 µg; channel e, BioGel A 1.5 M Fraction V, 0.7 µg.


a
b
c
d
e
phos
bsa
ov
chy
lys

purified fraction was used (Table 2-2). The specific activity of the ATPase was 150,000 units/mg.

ATP-Dependent Reannealing Activity

After chromatography on phosphocellulose, DNA reannealing was reproducibly achieved without contamination of nonspecific binding activity. The kinetics of reannealing in the presence and absence of ATP are illustrated in Figure 2-3. The level of reannealing achieved both as a function of time and protein concentration was approximately 40%. Although this level of activity is somewhat low, it is consistent with the amount of reannealing observed when β protein from phage λ was used under the same reaction conditions (Kmiec and Holloman, 1981) (see Chapter One). Therefore, the level of reannealing obtained in both these studies may be a reflection of the aggregation properties of the P22 DNA (Weinstock et al., 1979).

Homologous Pairing of Single Strand Fragments with Superhelical DNA

The formation of a triple stranded DNA structure by the uptake of a homologous DNA fragment by a superhelical molecule is thought to be an important part of the recombination pathway. Various studies have shown that the recA protein can use ATP as an energy cofactor to unstack the bases of the duplex while assimilating the homologous single strand fragment (Shibata et al., 1979; Cunningham et al., 1979). The D-loop structure thus formed contains both double and single stranded DNA regions. To assay for the formation of such structures, nitrocellulose filters were used because of their inherent quality of retaining single but not double stranded DNA (Beattie et al., 1977). The Ustilago protein catalyzed the formation of D-loops in a manner dependent on homology and, to a lesser extent, on ATP (Fig. 2-4).

TABLE 2-2

ATPase Activity Associated with the Ustilago Protein

DNA Cofactor	ADP Formed nmoles
single stranded ϕX174	5.50
ϕX174 form I	0.35
ϕX174 form II	0.36
none	< 0.20

Reactions were carried out as described in Appendix E. Individual mixtures contained 1 nmol of the indicated DNA and 30 ng of protein from Fraction V.

Fig. 2-3. Kinetics of reannealing.

A. Reaction mixtures (0.30 ml) containing 12 nmol denatured P22 [^{3}H]DNA, 6 µg of protein from fraction IV and ATP where indicated were incubated at 37° as described in Appendix E. Aliquots (50 µl) were removed at the times shown and the DNA resistant to hydrolysis by S1 was determined.

B. Reaction mixtures (50 µl) containing either 2 nmol denatured P22 [^{3}H]DNA or viral strand ϕX174 [^{3}H]DNA (3.2×10^4 cpm/nmol) were incubated with the indicated amount of protein from Fraction IV. DNA remaining susceptible to S1 was determined as described in Appendix E.

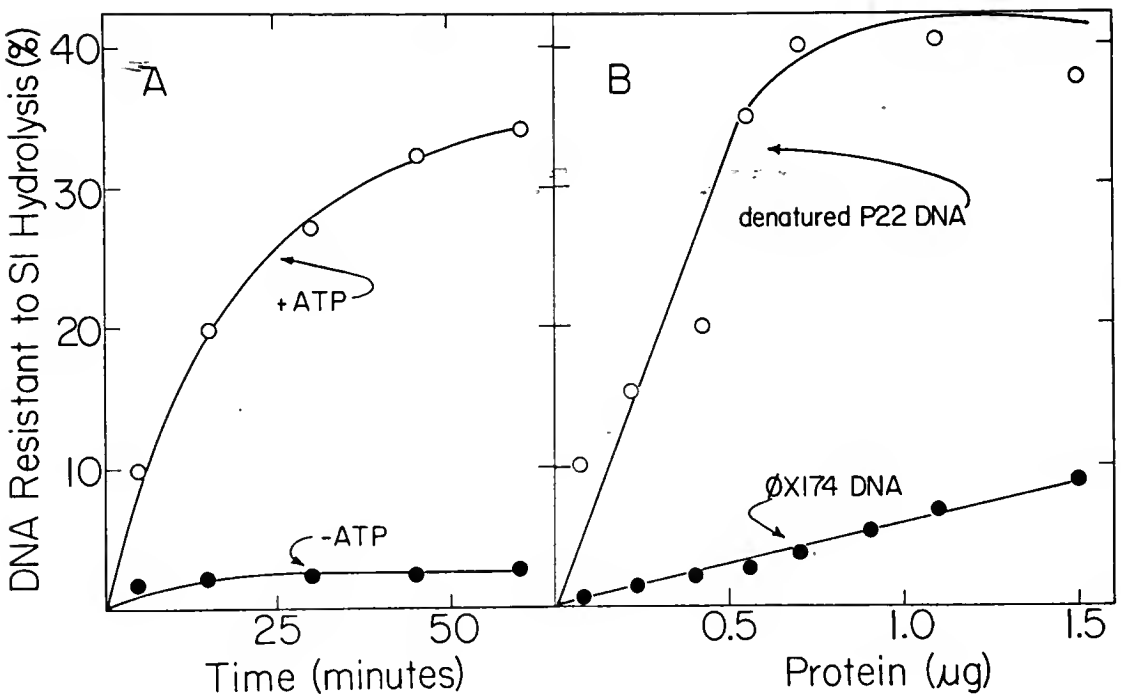
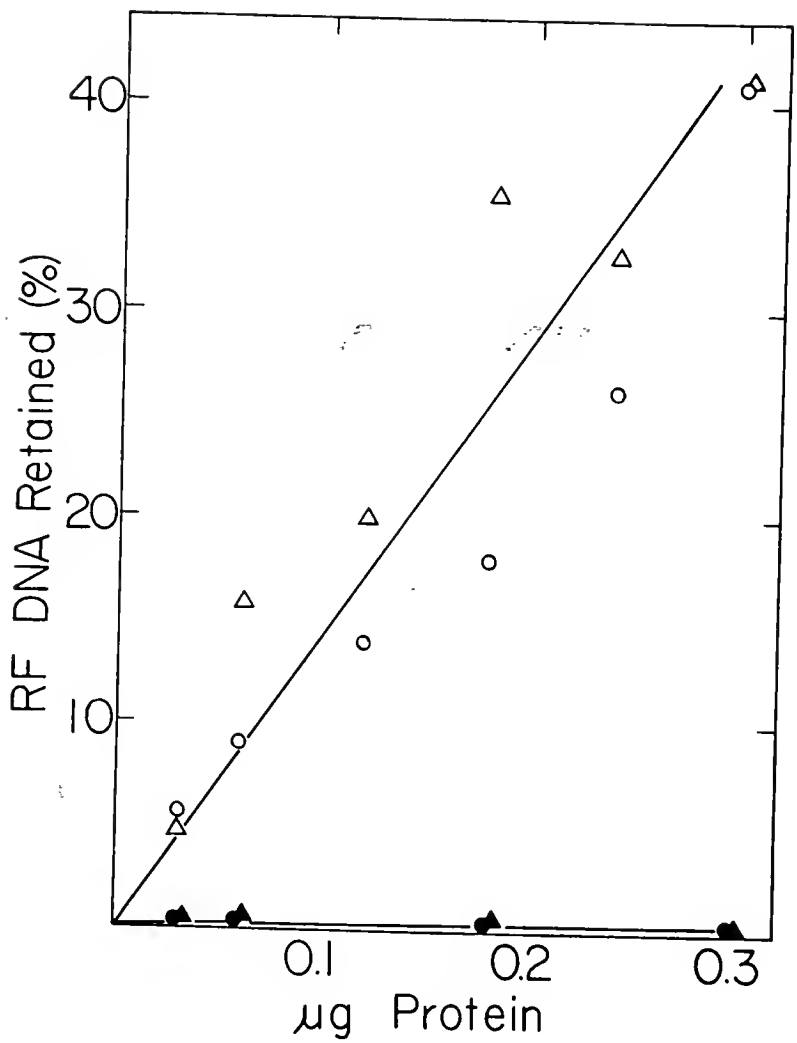

DNA Resistant to SI Hydrolysis (%)
40
30
20
10
A
+ATP
-ATP
25
50
Time (minutes)
B
denatured P22 DNA
ØX174 DNA
0.5
1.0
1.5
Protein (μg)

Fig. 2-4. Uptake of homologous single-strand fragments by superhelical DNA.

Reaction mixtures containing either ϕX174 RFI[^{3}H]DNA (2.0 x 10^4 cpm/nmol) or fd RFI[^{3}H]DNA (1.0 x 10^4 cpm/nmol) and the homologous or nonhomologous combination of single strand fragments were prepared and incubated with the indicated amount of protein from Fraction V. ϕX174 RF DNA was approximately 85% Form I and fd RF DNA was approximately 95% Form I as estimated by a nitrocellulose filter assay (Kuhnlein et al., 1976). ϕX RFI and ϕX fragments (△); fd RFI and fd fragments (○); ϕX RFI and fd fragments (▲); fd RFI and ϕX fragments (●).


40
30
20
10
RF DNA Retained (%)
0.1
0.2
0.3
µg Protein

Nonhomologous combinations were not effective substrates for the formation of joint molecules. A characteristic of joint molecules containing D-loops is that they are stable to 0.1% SDS or exposure to 75°C (Beattie et al., 1977). Over 90% of the D-loop molecules formed in reactions catalyzed by the Ustilago pairing protein were stable to either treatment.

Rec1 Mutant of Ustilago

A variety of phenotypically identifiable Ustilago mutants are available. Some of them, including the rec1 mutant, are deficient in certain types of recombination. The rec1 mutation and phenotype could be explained by loss of a regulatory function (Holliday et al., 1976). Because recA protein functions not only in homologous recombination but also in the regulation of cellular response to DNA damage (Clark, 1973), the rec1 mutant and two radiation-sensitive mutations were examined for altered levels of DNA pairing activity. As illustrated in Table 2-3, lower levels of DNA reannealing activity were observed in all the mutations. However, only in the rec1 mutant stain was the enzyme catalyzed DNA pairing activity diminished significantly. Therefore, the Ustilago pairing protein was designated rec1 protein.

Discussion

The experiments, outlined in this chapter, show that the lower eukaryote, Ustilago maydis, contains a protein which catalytically pairs DNA molecules in a reaction dependent on ATP. By using two assays which measure the reannealing of complementary single strands of DNA and the DNA-dependent hydrolysis of ATP respectively, the isolation and purification of this enzyme has been achieved. The uptake of single strand fragments by superhelical duplexes to form a

TABLE 2-3

Level of Reannealing Activity in Mutants

Strain haploids	Reannealing Activity units/mg
58 (*rec*+)	23,500
293 (*rec*1)	< 400
221 (*rec*2)	10,400
387 (*uvs*3)	20,600
diploids	
M133 (*rec*1-1 or *rec*1-2/+)	38,500
M133 S (*rec*1-1/*rec*1-2)	< 400

Cells were grown in two liters of medium and processed as described in Appendix D. Specific activities indicated are the values obtained after phosphocellulose chromatography.

three-stranded structure known as a D-loop can be catalyzed by recl protein. Most molecular models describing the mechanism of recombination include the formation of D-loops as a vital step (Meselson and Radding, 1975; Wilson, 1979). Taken together these observations suggest that the recl protein may function directly in recombination pathways. An interesting observation concerning the formation of D-loops is that ATP is not an absolute requirement for the reaction. For these lower levels of D-loop formation, the recl protein may utilize the energy of the superhelix, thereby bypassing the need for ATP.

Evidence for the role ATP plays in contributing an energy source for such pairing reactions catalyzed by recA protein is somewhat dichotomous. Early studies on strand uptake reactions indicated that ATP was an absolute requirement (Shibata et al., 1979). Recently, it has been reported that the energy generated by the hydrolysis of ATP is used as the enzyme dissociates from a D-loop molecules (Shibata et al., 1982a). The strand uptake reaction may rely at least in part on the energy of the superhelix (Shibata et al., 1982b).

The formation of joint molecules catalyzed by the recl protein occurs at a much lower stoichiometry than those reactions catalyzed by recA protein. Calculation based on D-loop formation data indicates that one recl monomer is required per 200 nucleotides of single stranded DNA (Kmiec and Holloman, 1982). The recA protein-DNA nucleotide ratio is approximately one to three (Shibata et al., 1979). Part of this difference may be attributed to the larger size of the recl protein (70,000 daltons). However, a more interesting possibility is that the recl protein may possess an enzymatic feature absent in the

recA protein. For example, the amount of recA protein required for pairing reactions can be substantially reduced if single strand binding protein is present (Soltis and Lehman, 1983). Perhaps the characteristic reaction requisite of SSB in pairing reactions promoted by recA protein is inherent in the recl protein.

Mutations in the recl gene produce phenotypes are deficient in recombination, repair and cell division (Holliday et al, 1976). Because of the similarities to recA mutant phenotypes (Clark, 1974; Gottesman, 1981; Roberts et al., 1978), it is attractive to postulate a role for the recl protein in certain cellular regulation pathways. At this time no regulatory functions, such as an endopeptidase activity, has yet been associated with the recl protein. However, in the absence of a specific substrate, such studies are particularly difficult.

Recombinational deficiencies in the recl mutation include a lack of gene conversion events (Holliday, 1967). Although the molecular basis for gene conversion is not completely understood, it is generally believed that a heteroduplex molecule is first created followed by a correction process known as mismatch repair. The formation of this heteroduplex molecule is nonreciprocal and therefore may begin via an asymmetrical exchange of DNA strands. The formation of D-loops catalyzed by the recl protein in vitro is an asymmetric exchange reaction.

CHAPTER THREE
HETERODUPLEX FORMATION PROMOTED BY USTILAGO REC1 PROTEIN

The formation of a heteroduplex joint is a key feature in the overall process of reciprocal recombination. This structure, a hybrid molecule consisting of complementary DNA strands from different parents, has also been applied to the processes of bacterial conjugation (Lacks, 1970). The biochemical and molecular events preceding heteroduplex formation are becoming more evident due mainly to the characterization of the properties of the recA protein from E. coli (Weinstock et al., 1979; Shibata et al., 1979). It has previously been reported that the lower eukaryote Ustilago maydis contains a protein which promotes the pairing of a variety of homologous DNA substrates in vitro (Kmiec and Holloman, 1982). One of these pairing reactions involves the uptake of a single strand fragment by a superhelical duplex. The joint molecule formed, known as a D-loop, resembles a molecular intermediate postulated to exist in an array of recombination models (Meselson and Radding, 1975; Wilson, 1979). However, this particular strand transfer reaction is somewhat difficult to quantitate.

Circular, single stranded DNA and linear duplex DNA constitute a pair of substrates potentially useful in studying the pairing reaction. This reaction has the advantage that the substrates and the products can be easily identified and quantified by a variety of appropriate

assays. Recent experiments in several laboratories have led to the conclusion that the recA protein efficiently transfers a single strand circle onto a linear duplex in a reaction coupled to the hydrolysis of ATP (Cox and Lehman, 1981; Cox et al., 1982). The recA protein-promoted DNA strand transfer reaction occurs in two, experimentally distinguishable, phases (Cox and Lehman, 1981). The first, known as synapsis brings the two DNA molecules into homologous alignment (Wu et al., 1982; Gonda and Radding, 1983). This structure can be measured by a filter-binding assay using nitrocellulose filters in which duplex DNA is retained as a function of its single strandedness (Beattie et al., 1977). The reaction requires ATP, but not its hybrolysis and stoichiometric amounts of recA protein (Cox and Lehman, 1981). Synapsis can take place in the absence of any homologous free ends, although stable hybrid molecules cannot form under these conditions (Wu et al., 1983). Topological linkage of such paired molecules by the use of topoisomerase can produce stable joint molecules called hemicatenanes in which the circular single strand is interwound with its complementary strand (Cunningham et al., 1981).

The second phase of the strand transfer reaction is known as strand exchange or branch migration. Whereas synapsis occurs quickly, the assimilation of the circle and the concurrent displacement of its homolog-strand in the duplex is slow. The branch migration reaction can be measured using the nuclease, S-1, which digest the displaced strand of the duplex and therefore is a true measure of heteroduplex formation. By using this assay, a variety of investigators have determined that the strand exchange process follows sigmoidal kinetics (Cox and Lehman, 1981). Furthermore, recA protein promoted branch

migration occurs in a 3' to 5' polarity relative to the minus strand (Kahn et al., 1981). Presumably, synapsed molecules formed at the "unfavored end" of the linear duplex are quickly eliminated by interaction with ADP (Wu et al., 1982).

RecA-protein promoted strand exchange is stimulated 5-fold to 20-fold by single-strand binding protein (SSB) (Cox and Lehman, 1982). In the presence of SSB and ATP, the binding of single stranded DNA by recA-protein is stabilized and the complex, therefore, is more able to interact with duplex DNA (Cox and Lehman, 1982; Soltis and Lehman, 1983). This stimulation alters the rate-determining phase of DNA strand exchange.

The branch migration phase of strand exchange requires the concurrent hydrolysis of ATP (Cox and Lehman, 1981). Approximately, 10 to 15 ATP molecules are hydrolyzed per heteroduplex base pair (Cox et al., 1982). ATP hydrolysis actually reflects the dissociation of the recA protein from the heteroduplex molecule (Ohtani et al., 1982). How chemical energy is assimilated and utilized in the strand transfer reaction is currently unknown.

Since the rec1 protein from Ustilago maydis carries out strand transfer reactions similar to those described for the recA protein, it is particularly important to test the eukaryotic protein under such in vitro recombination criteria. A more detailed understanding of the mechanisms of strand transfer will lead to a fuller appreciation of heteroduplex formation promoted by rec1 protein.

Initiation of Strand Transfer

The formation of joint molecules can be measured in two ways. The first is the assay used to detect D-loops; that is binding to

nitrocellulose filters in washes of high salt (see Chapter Two). The second involves the sensitivity of S-1 nuclease digestion of a displaced strand of DNA during the assimilation of a single strand circle. When circular single strands of DNA were incubated with homologous linear duplex in the presence of recl protein and ATP, joint molecule formation was evident using either assay. The filter binding assay showed that the formation of joint molecules is immediate and increase in a linear fashion (Fig. 3-1A). However, the kinetics of joint molecule formation as measured by the S-1 nuclease assay are different. In this assay the labeled DNA molecule is the circular single strand and the determination is the development of S-1 nuclease resistance as a function of time. Here sigmoidal kinetics are observed (Fig. 3-1A). After a lag of several minutes, a rapid rise in the labeled DNA resistant to S-1 is seen. Yet the maximum level of joint molecules formed was the same in both assays. The initial high rate of joint molecules formation, detected by the nitrocellulose filter assay may indicate that formation of joint molecules can occur in the absence of extensive heteroduplex.

Heating the reaction mixtures prior to filtration eliminates a large fraction of the joint molecules formed early in the reaction (Fig. 3-1B). The formation of heat-stable joint molecules follows sigmoidal kinetics.

Joint molecule formation can also be measured by agarose gel electrophoresis (Fig. 3-2). During early reaction times, no product molecules are seen (lanes d-i). However, after 20 minutes of incubation a band appeared which migrated near the form II DNA marker (lane a, upper band). Subsequent joint molecule formation occurred

Fig. 3-1. Formation of joint molecules assayed by two different methods.

A. Reaction mixtures of 0.5 ml contained 15 µg/ml rec1 protein and either 15 µM ^{3}H-labeled fd form III DNA (1.2×10^{4} cpm/nmol) and 7.5 µM fd circular single stranded DNA or 15 µM fd form III DNA and 7.5 µM ^{3}H-labeled fd circular single stranded DNA (1.6×10^{4} cpm/nmol). Aliquots of 40 µl were removed at the indicated times and formation of joint molecules was measured by retention on nitrocellulose filters (o) or by resistance to S-1 nuclease (•).

B. Aliquots from a reaction mixture as in A were held at 50°C or 37°C for 4 minutes before filtering through nitrocellulose. The ratio indicates the fraction of joint molecules surviving treatment at 50°C compared with that at 37°C.

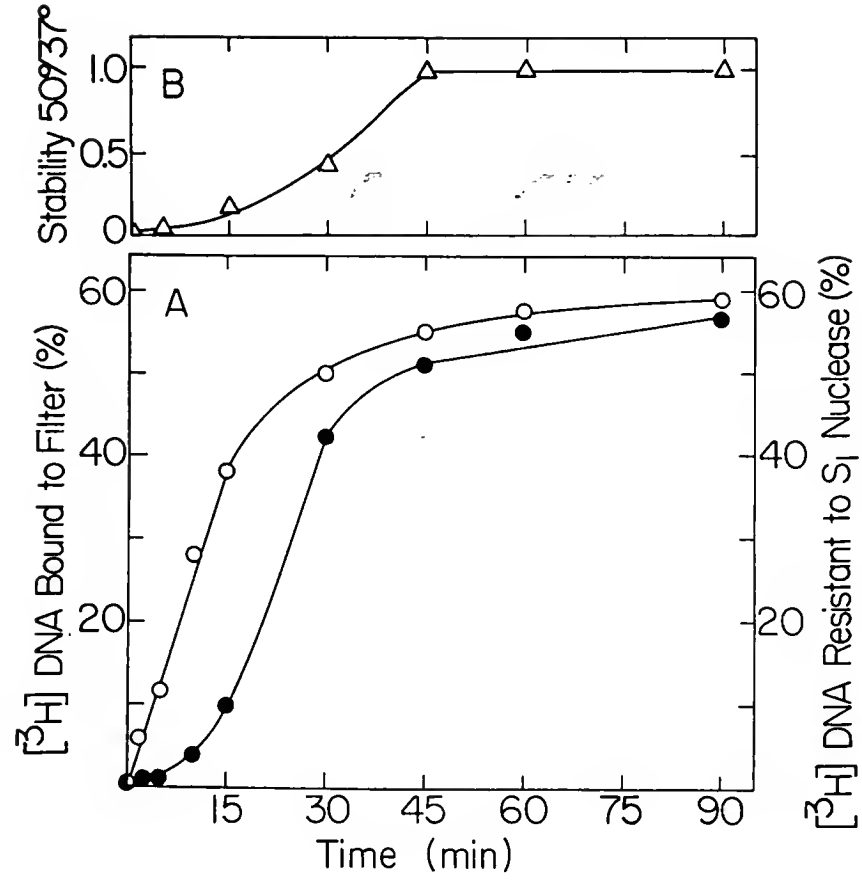
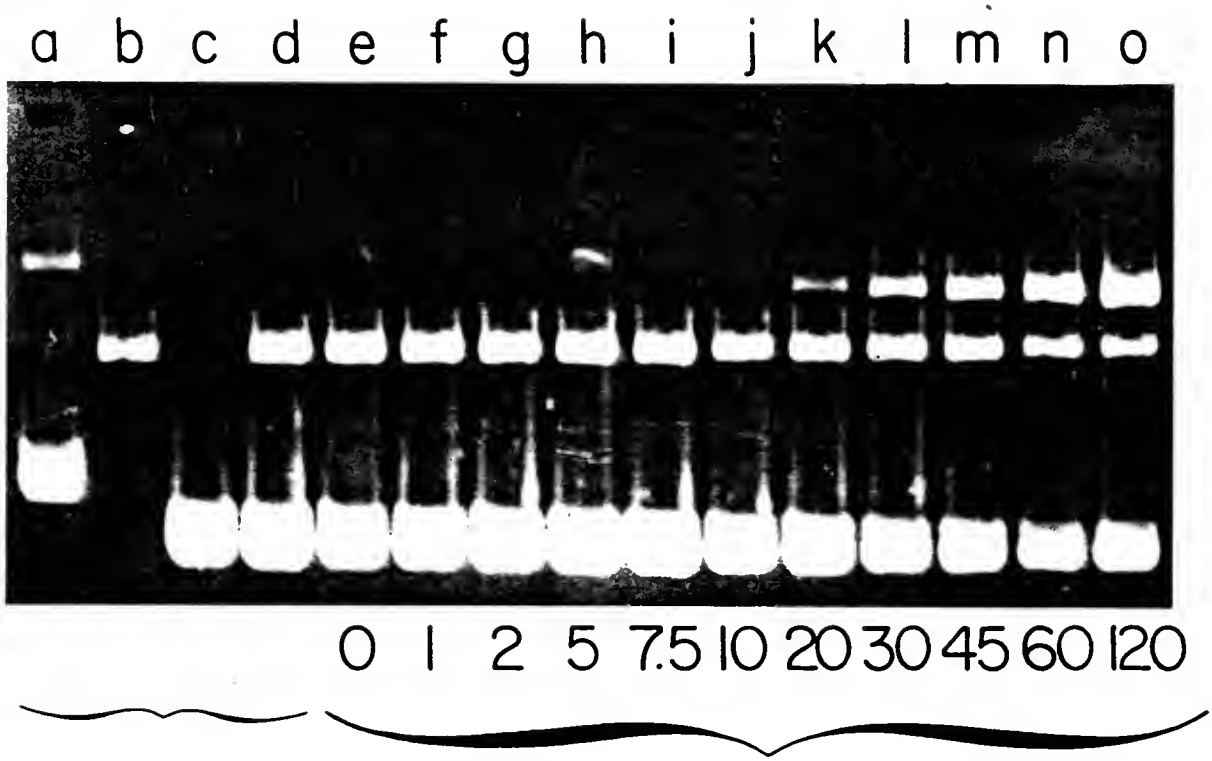

Stability 50°/37°
1.0
0.5
0
B
A
60
40
20
[3H] DNA Bound to Filter (%)
[3H] DNA Resistant to S1 Nuclease (%)
15
30
45
60
75
90
Time (min)

Fig. 3-2. Formation of joint molecules measured by agarose gel electrophoresis.

A reaction mixture of 0.32 ml contained 15 μM fd form III DNA, 7.5 μM fd circular single stranded DNA, and 15 μg/ml rec1 protein. Aliquots of 40 μl were removed at the indicated times, mixed with 10 μl of a solution containing 0.25% bromophenol blue, 0.5% SDS, 25 mM EDTA, and 20% glycerol, loaded into slots of an agarose gel and electrophoresed as described. a) fd form I DNA contaminated with a small amount of slower moving form II DNA, b) fd form III DNA, c) fd circular single stranded DNA, d) reaction mixture minus rec1 protein, e-o) course of reaction at indicated times (minutes).


a b c d e f g h i j k l m n o
0 1 2 5 7.5 10 20 30 45 60 120
Controls
Time Course

after 20 minutes of incubation (lanes l-o). The formation of a form II-like DNA molecule and the formation of extensive heteroduplexed DNA correlate quite well as a function of time.

Strand transfer reactions required the presence of ATP, although the nonhydrolyzable ATP analog adenylyl-imidodiphosphate (AMPPNP) could serve as a adequate substitute when product molecules were measured by the filtering-binding assay (Kmiec and Holloman, 1983). The level attained using AMPPNP in place of ATP was three- to fourfold lower, and the complexes formed in such reactions were unstable and did not require homologous partners.

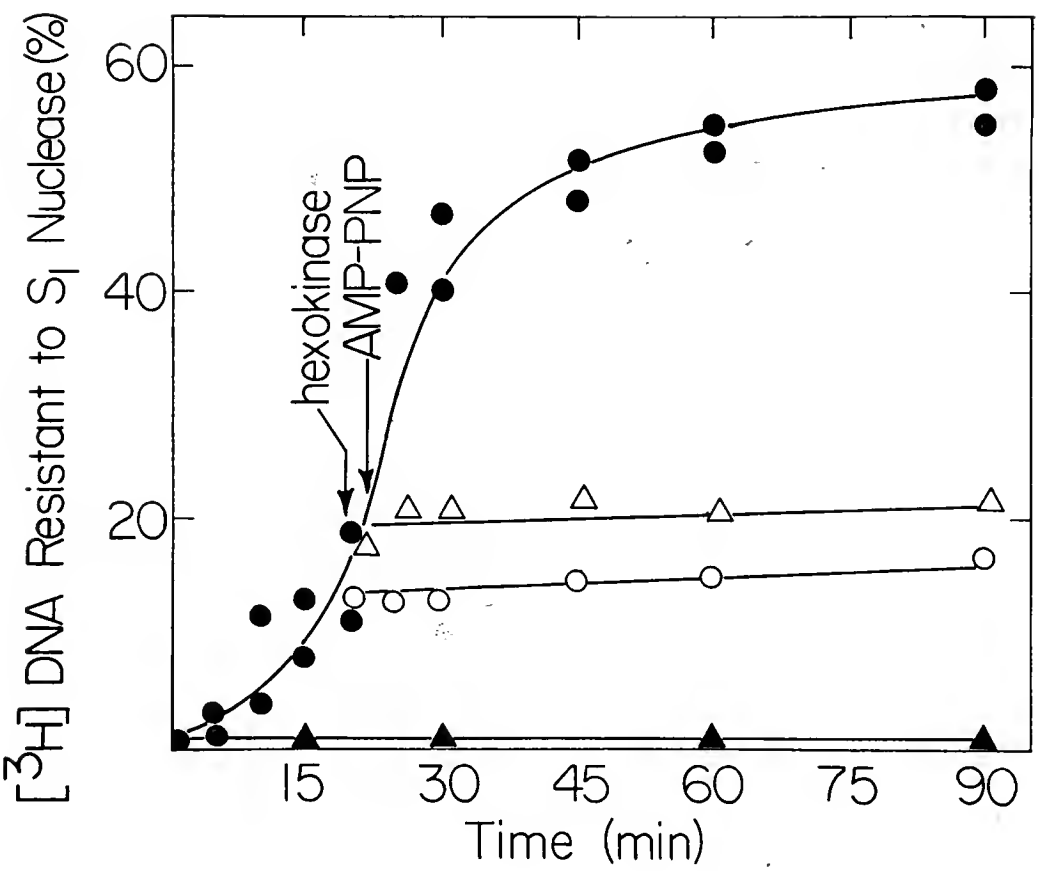
Energy Requirements for Heteroduplex Growth

Extention of the initiation complex joint required ATP hydrolysis. When AMPPNP was added to an ongoing reaction, strand exchange stopped immediately (Fig. 3-3). The addition of glucose and the enzyme hexokinase, which catalytically converts ATP to adenosine diphosphate (ADP) in a rapid manner, also stopped the strand transfer reaction (Fig. 3-3). This continual requirement for ATP in its hydrolyzable form suggests that its energy is used by rec1 protein to drive strand exchange reactions.

Mechanism of Strand Transfer

The formation of a heteroduplex joint molecule involves strand transfer of the single strand circle into the duplex linear molecule. As the circle is assimilated, the homolog duplex strand would be displaced. Does the displacement of this strand occur in a preferred direction? That is, is there a favored end to start the strand exchange reaction and is there a subsequent polarity to heteroduplex formation?

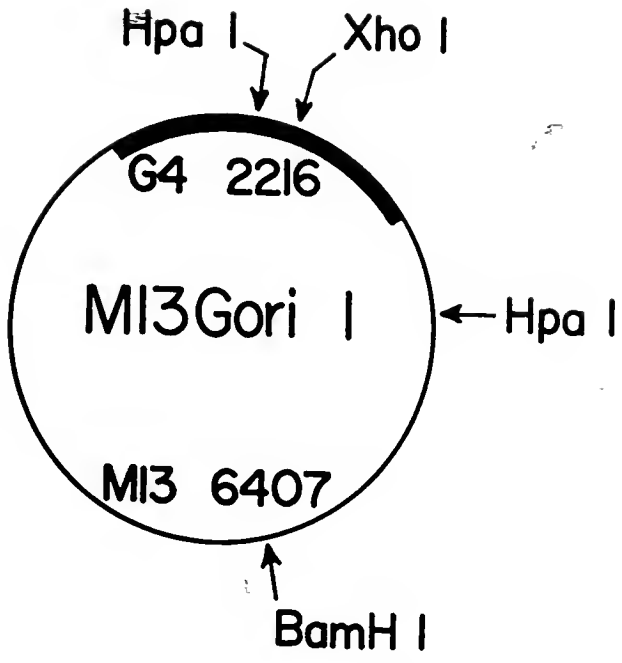
Fig. 3-3. ATP requirement in formation of joint molecules.
Reaction mixtures of 0.4 ml contained 15 µg/ml rec1 protein, 15 µM ^{3}H-labeled fd form III DNA (1.2×10^{4} cpm/nmol) 7.5 µM circular single stranded DNA and either ATP (●), adenylyl-imidodiphosphate (○), adenylyl-imidodiphosphate and G4 circular single stranded DNA in place of fd circular DNA (△), or no ATP (▲). Aliquots of 40 µl were removed and formation of joint molecules was measured by the nitrocellulose filter assay.


[3H] DNA Resistant to S1 Nuclease (%)
60
40
20
hexokinase
AMP-PNP
15
30
45
60
75
90
Time (min)

To first explore the role of ends in the pairing of single strand circle and linear duplexes, DNA from the chimeric phage M13 Gori1 was used (Kaguni and Ray, 1979) (Fig. 3-4). This phage consists of 2216 bases of G4 inserted into the M13 phage genome. A single XhoI restriction site exists in the G4 region, while the M13 region contains a unique BamHI cleavage site. When M13 Gori1 duplex linear, cleaved by the enzyme XhoI, was used in reactions containing rec1 protein and G4 single stranded circular DNA, high levels of joint molecules were formed (Fig. 3-5). However, under the same reaction conditions, but substituting fd single strand circles very low levels of joint molecules were formed (Fig. 3-5A). Phage fd is 97% homologous to the M13 DNA sequence (van Wesenbeck et al., 1980). The reverse reaction using BamHI restriction of M13 Gori1 DNA which contains M13 sequences at its termini produced joint molecules only when fd single strand circles were used (Fig. 3-5B).

These results emphasize the importance of ends in the production of joint molecules stable under reaction conditions which involved washes of high salt (see Appendix E). However, this does not suggest that the strand transfer reaction begins at a unique, preferred end. To investigate this possibility, duplex linear molecules were made as described by Kahn et al. (1981). In this procedure duplex linear molecules complementary to the (+) viral circular single strands at either the 5' or the 3' end can be created. The restriction enzyme HpaI cuts M13 Gori1 DNA in two sites. One site is located within the M13 region, the other in the G4 sequence. The cut within the G4 sequence produces a fragment 6583 base pairs long with G4 DNA at the 5' end of the minus strand. The cut within the M13 region produces a

Fig. 3-4. Restriction maps of DNA from phages M13 Gori1 and fd.
Sites of cleavage by restriction endonucleases HpaI, XhoI, BamHI, and Sau96I have been redrawn from the maps shown by Kahn et al. (1981) and Cunningham et al. (1981).


Hpa I
Xho I
G4 2216
M13Gori I
Hpa I
M13 6407
BamH I

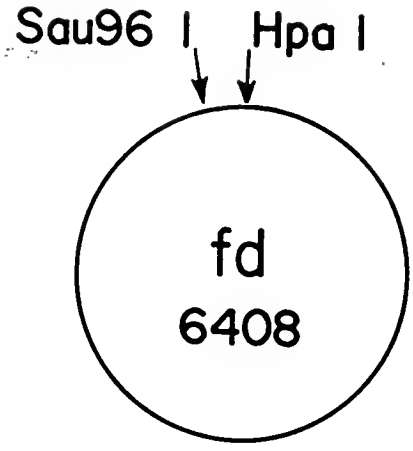
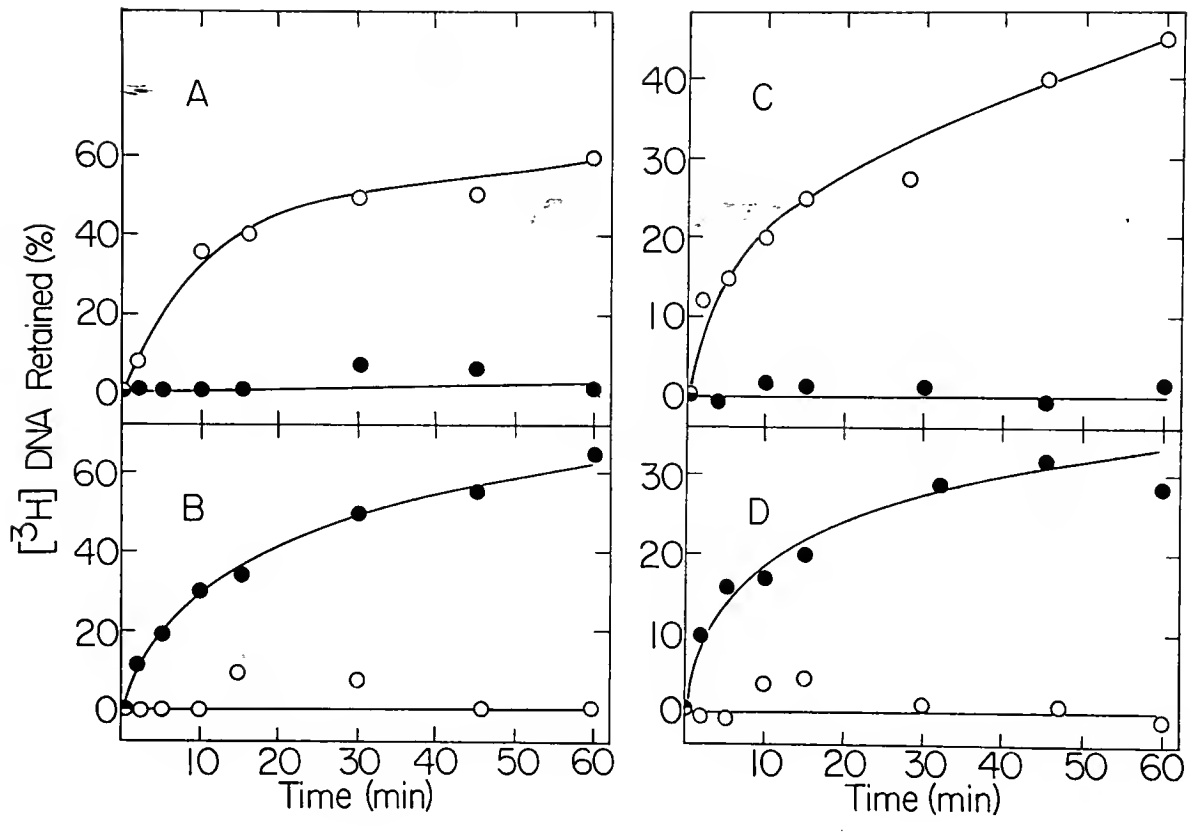

Sau96 I
Hpa I
fd
6408

Fig. 3-5. Formation of joint molecules at a preferred end.

Since the sizes of linear duplex molecules and circular single stranded molecules varied from reaction to reaction in this experiment, concentrations indicated refer to moles of the full length molecule. Calculations were based on the following sizes: 8623 base pairs, M13 Gori1 (Kaguni and Ray, 1979); 6583 base pairs, M13 Gori1 HpaI large fragment (Kahn et al., 1981); 2040 base pairs, M13 Gori1 HpaI small fragment (Kahn et al., 1981); 6408 bases, fd DNA (Beck et al., 1978); 5577 bases, G4 DNA (Godson et al., 1978). A. Reaction mixtures of 320 µl contained 0.6 nM ^{3}H-labeled M13 Gori1 form III DNA (prepared with XhoI) and either 0.6 nM G4 or fd circular single strands, plus 12 µg/ml recl. B. Reaction mixtures of 320 µl contained 0.6 nM ^{3}H-labeled M13 Gori1 form III DNA (prepared with BamHI) and either 0.6 nM G4 or fd circular single strands plus 12 µg/ml recl. C. Reaction mixtures of 320 µl contained 0.6 nM ^{3}H-labeled M13 Gori1 DNA HpaI large fragment, and either 0.6 nM G4 or fd circular single strands, plus 6 µg/ml recl. D. Reaction mixtures of 320 µl contains 0.6 nM ^{3}H-labeled M13 Gori1 DNA HpaI small fragment, and either 0.6 nM G4 or fd circular single strands, plus 6 µg/ml recl. Aliquots of 40 µl were removed at the indicated times and joint molecules were determined using nitrocellulose filter assay. (○) G4 DNA, (●) fd DNA.


A
B
C
D
[3H] DNA Retained (%)
Time (min)
Time (min)

fragment 2040 base pairs long with M13 DNA at the 5' end of the minus strand. When the larger fragment was reacted with either G4 or fd single stranded circles, joint molecules were formed only with the G4 single stranded circles (Fig. 3-5C). The fd single stranded circles paired only with the smaller duplex fragment (Fig. 3-5D). Taken together these results indicate that single stranded circular DNA is transferred onto a homologous linear duplex to create a stable heteroduplex by pairing with the 5' end of duplex complementary strand. Accompanying the base pairing of the circle with the 5' end of the duplex minus strand is the concurrent displacement of the 3' end, and subsequently the entire length of the positive duplex strand (Kmiec and Holloman, 1983).

Discussion

The rec1 protein catalyzes the homologous pairing of circular single stranded and linear duplex DNA in a reaction occurring in at least two distinguishable phases. The first involves synapsis of the two DNA molecules with the alignment of homologous sequence, but little heteroduplex formation. The reaction is dependent on the presence of ATP as well as DNA homology. In the second phase of the reaction, strand exchange, the nascent heteroduplex lengthens to form a stable joint molecule. This progressive branch migration is slower than synapsis, requires ATP hydrolysis and occurs in a polar direction. The mechanism by which the two DNA molecules become homologously aligned is currently unknown. In recA protein promoted reactions, the enzyme polymerizes first on single stranded DNA then processively searches the recipient duplex molecule for complementary sequences (Gonda and Radding, 1983). Once aligned the formation of heteroduplex DNA

proceeds in a 3' to 5' polar direction, with respect to the minus strand of the duplex (Kahn et al., 1981; West et al., 1982). Remarkably, its direction is opposite to that of recl protein-promoted strand transfer reactions.

The polar movement of strand transfer reactions may be a function of the recl protein's unwinding activity. Although no direct evidence has been produced showing that this enzyme unwinds duplex DNA, it can be assumed that this activity takes place prior to homologous pairing in D-loop formation. There are a number of DNA enzymes whose activity involves polar motion in relation to the duplex DNA. These include DNA polymerases and in particular, DNA helicases (Yarrington and Gefter, 1979).

The significance of the polar movement inherent in heteroduplex formation reactions promoted by recl protein in vitro is unclear. In meiotic recombination, heteroduplex DNA, once formed seems to grow in a polar fashion (Rossignol et al, 1978). In fungi, polarity in recombination events has been reported (Catcheside and Angel, 1974). In yeast there is evidence indicating that recombination begins at a defined site and extends into a locus from a particular direction (Fogel et al., 1978).

In recA-protein promoted strand exchange reactions, formation of D-loops, nascent heteroduplexes, does not exhibit a polarity (Cox and Lehman, 1981). Wu et al. (1983) found that nascent heteroduplexes may occur at either end of the linear duplex molecule, but under the regulatory mechanism of ADP only those at the favored end persist, eventually elongate into a stable recombinant molecule. The control of heteroduplex formation promoted by recl protein may also involve ADP

since this molecule is an inhibitory reaction component to the extension of heteroduplex joints.

CHAPTER FOUR
TOPOLOGICAL LINKAGE OF CIRCULAR MOLECULES CATALYZED BY REC1 PROTEIN AND TOPOISOMERASE

It is currently believed that the mechanism of genetic recombination involves the breakage and reunion of homologous chromosomes (Taylor, 1965). This concept poses a fundamental and still largely unanswered question. Does recognition of homology precede breakage or follow it? To produce the cross-stranded structure, known as a Holliday structure (1964), molecular events have been proposed which, by and large, embody single strand breaks (Hotchkiss, 1974; Meselson and Radding, 1975), because generation of single strand ends prior to synapsis avoids topological problems. Furthermore, free single strand (Benbow et al., 1974) and double strand (Szostak et al., 1983) ends have been observed to be recombinogenic. The discovery of a group of enzymes known as topoisomerases which can relieve the topological constraints of interwound DNA molecules (Kirkegaard and Wang, 1978) have strengthened the theory that pairing precedes breakage (Wilson, 1979; Kikuchi and Nash, 1979).

The rec1 protein from Ustilago maydis pairs single stranded circular DNA with homologous linear duplex in a two step reaction. The first, known as synapsis, conjoins homologous DNA sequences while the second, strand exchange, slowly increases the heteroduplex length (Chapter 3; Kmiec and Holloman, 1983). In those studies, the importance of homologous free ends in the creation of stable heteroduplex molecules was established. However, the possibility still existed that

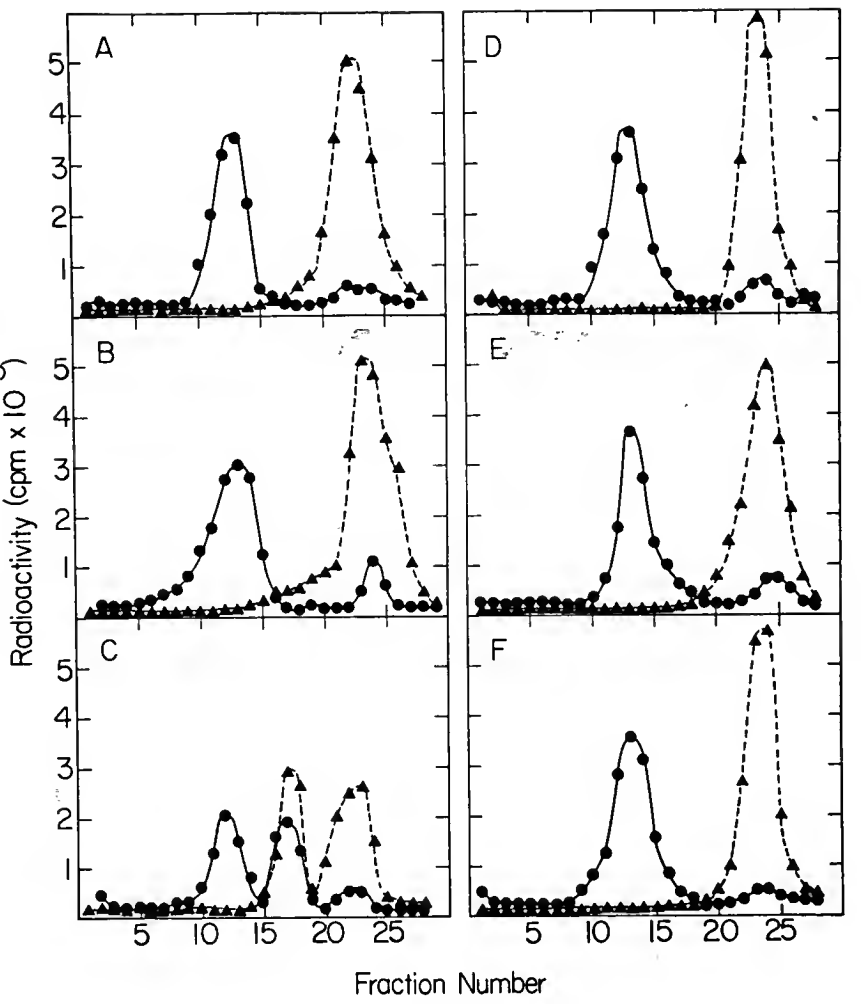
synapsis began in regions of homology lacking free ends and then incorporated free ends to increase the stability of the joint molecules. The synapsis of the DNA molecules and the search for homology comprise a set of reactions which *in vitro* correlate with the initiation of homologous reciprocal recombination *in vivo*. To explore this possibility, experiments were designed to test the ability of recl protein to homologously pair molecules lacking free ends. Such a synapsed pair of molecules would be subject to catenation by the enzyme, topoisomerase. Successful enzymatic pairing of two circular homologous molecules followed by topological linkage would demonstrate that during the initiation of recombination pairing precedes breakage.

Homologous Pairing and Topological Linkage of Single Stranded Circles and Closed Circular Duplex DNA

E. coli recA protein has been observed to pair single stranded DNA with homologous duplex DNA in a side-by-side fashion (Wu *et al.*, 1983). Previously, Cunningham *et al.* (1981) showed that molecules paired in a side-by-side manner could be topologically linked after addition of *E. coli* topoisomerase I. Circular single stranded DNA, homologous superhelical DNA, ATP and recl protein were incubated together for 45 minutes. This was followed by addition of Ustilago topoisomerase I (Rowe *et al.*, 1981) and the product molecule identified by sedimentation in gradients of alkaline sucrose. As illustrated in Figure 4-1C, linked molecules comprised of [^{3}H]-labeled superhelical duplex DNA and [^{32}P]-labeled single stranded circles were observed to sediment between each of the parent molecule populations. No product was observed in reactions lacking recl protein, ATP or topoisomerase (Fig. 4-1A, B, D). Nonhomologous combinations of DNA were ineffective

Fig. 4-1. Linkage of single-stranded circles and form I DNA.

Complete reaction mixtures of 100 µl contained 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothrietol, 10 µM ^{3}H-labeled fd form I DNA, 10 µM ^{32}P-labeled fd circular single-stranded DNA, and 40 µg/ml rec1 protein. After 45 minutes at 37°C 20 units of topoisomerase were added and incubation was continued for an additional 30 minutes. The reactions were stopped by addition of EDTA to 0.1 M and NaOH to 0.3 M. Mixtures were layered on top of alkaline sucrose gradients and the gradients centrifuged for 2 hours at 45,000 rpm in a Beckman SW50.1 ti rotor. (A) minus rec1 protein (B) minus topoisomerase (C) complete reaction (D) minus ATP (E) ^{32}P-labeled ϕX174 DNA (2.8×10^4 cpm/nmol) in place of fd single stranded DNA (F) control, DNA alone. (●) ^{3}H-labeled DNA. (▲) ^{32}P-labeled DNA.


A
B
C
D
E
F
Radioactivity (cpm x 10^{-3})
Fraction Number
5
10
15
20
25
1
2
3
4
5

substrates for joint molecule formation (Fig. 4-1E). The sedimentation pattern observed for the product joint molecule is expected due to the increase in the frictional coefficient of the catenane.

To analyze the structure of the product made when circles and superhelical duplexes are linked, we once again employed the chimeric phage M13 Gori1 (Kaguni and Ray, 1979). Using [^{32}P]-labeled G4 phage DNA and [^{3}H]-labeled M13 Gori1 superhelical duplex DNA (form I), rec1 protein and topoisomerase acting in concert created joint molecules topologically linked within the G4 stretches of the M13 Gori1 DNA as determined by alkaline sucrose gradients. Approximately 27% of the input M13 Gori1 form I DNA became complexed with G4 phage DNA. Restriction enzyme digests of the joint molecule, after isolation and renaturation, within the G4 region of M13 Gori1 dissociated the complexes. Restriction enzyme digests inside the unpaired M13 region did not dissociate the complexes. Therefore, the structure of the joint molecules containing single stranded circles and superhelical duplex is a hemicatenane (Cunningham et al., 1981) in which the circle is linked to the duplex through interwinding with its complement in the duplex (Kmiec et al., 1983). When the extent of the heteroduplex formation was measured by S-1 nuclease digestion approximately 32% of the input G4 single strand circle became resistant (Table 4-1). This amount is equivalent to the formation of a length of 1780 base pairs, accounting for nearly the entire length of the G4 insert in M13 Gori1 DNA.

Reaction Requirements

Since the energy from superhelix formation or from ATP hydrolysis might be expected to drive the catenation, reactions were carried out

TABLE 4-1

Extent of Heteroduplex Formation

Enzymes Added		DNA Resistant to S-1 Digestion	
Topoisomerase	Rec1 Protein	%	Length in Nucleotides
+	-	<0.5	<30
-	+	2.0	110
+	+	32.0	1780

Reaction mixtures of 100 µl containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP, 50 µM M13 Gori1 form I DNA (8 x 10^3 cpm/nmol), 25 µM G4 single stranded circular DNA (2 x 10^4 cpm/nmol), and 15 µg/ml rec1 protein were incubated at 37°C. After 20 minutes 200 units/ml topoisomerase was added and incubation continued for 15 minutes. The reaction was stopped by addition of 0.4 ml of 0.2 M sodium acetate buffer pH 4.5, 2 mM $ZnCl_2$ and 20 units of S-1 nuclease, incubation was continued for 30 minutes, then 0.25 ml of 1 mg/ml carrier DNA and 0.25 ml of 10% trichloroacetic acid were added. The precipitates were washed onto Whatman GF/C glass fiber filters and the filters washed with 10 ml of 5% trichloroacetic acid, 2 ml ethanol, then dried and the ^{32}P radioactivity bound was determined.

using relaxed closed circular duplex DNA and the nonhydrolyzable analog adenylyl-imidodiphosphate. Form I DNA, relaxed by topoisomerase and freed of protein by phenol extraction, was an effective substrate for catenation (Fig. 4-2B). The ATP analog could substitute as a cofactor in the reaction, although the hemicatenanes were formed without regard to homology (Fig. 4-2C). This result concurs with earlier observations (Chapter Three) which indicated that joint molecules could form without regard to homology when the analog was used (determined by the filter binding assay).

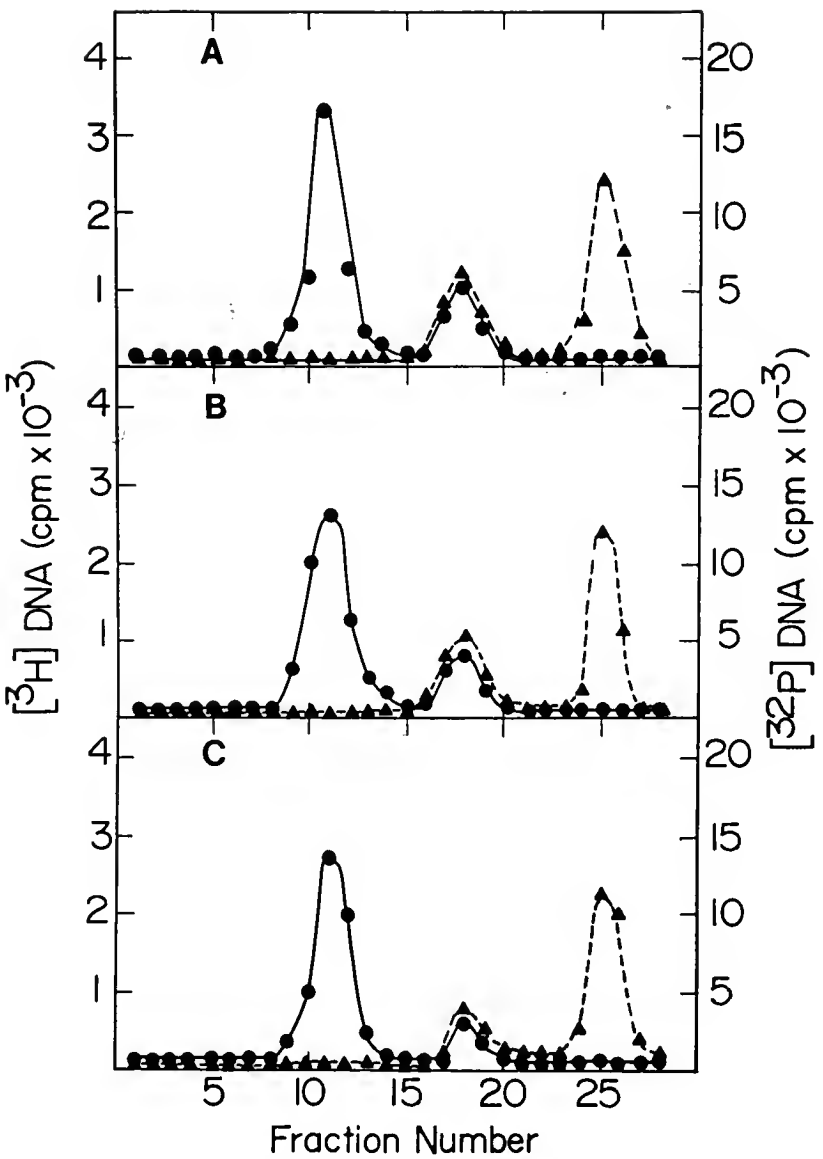
Catenation of Intact Homologous Duplex DNA Molecules

Circular duplex molecules can also be linked, albeit, independent of homology by type I topoisomerases (Tse and Wang, 1980; Brown and Cozzarelli, 1981). These joined molecules are catenated like links in a chain (full catenane; contrasted with hemicatane). Homology dependent topological linkage of duplex DNA molecules has not been reported. When [^{32}P]- and [^{3}H]-labeled M13 superhelical DNA was reacted with recl protein, ATP and topoisomerase either concurrently or added in succession, no catenated molecules were observed to form.

Previously, it was shown that recl protein catalyzes the uptake of a homologous single strand fragment by superhelical DNA to form a D-loop structure (Kmiec and Holloman, 1982). Since this joint molecule could be an intermediate in recombination and a possible starting point for crossover junctions between two duplexes, an experiment was designed in which D-looped DNA duplex was catenated with superhelical duplex DNA. D-loop DNA can be prepared by either thermal annealing (Beattie et al., 1977) or by recl protein catalysis. [^{3}H]-labeled and [^{32}P]-labeled fd form I DNA was reacted with recl protein and

Fig. 4-2. Linkage of single stranded circles with duplex circular DNA in the absence of homology or superhelicity.

Reaction mixtures of 100 µl containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP or adenylyl-imidodiphosphate, 10 µM ^3H-labeled fd form I DNA or form IV DNA (form I relaxed with topoisomerase), 10 µM ^{32}P-labeled fd or G4 single-stranded circular DNA, and 40 µg/ml rec1 protein were incubated at 37°C for 20 minutes. After topoisomerase was added to 200 units/ml incubation was continued for 20 minutes. Reactions were stopped by addition of 50 µl 0.25 M EDTA and 5 µl 10 M NaOH. Mixtures were loaded on alkaline sucrose gradients and centrifuged at 45,000 rpm for 2 hours in a Beckman SW50.1 ti rotor. Reaction mixtures contained (A) fd circles, fd form I DNA and ATP (B) fd circles, fd form IV DNA and ATP (C) G4 circles, fd form I DNA, and adenylyl-imidodiphosphate. (●) ^3H-labeled DNA. (▲) ^{32}P-labeled DNA.

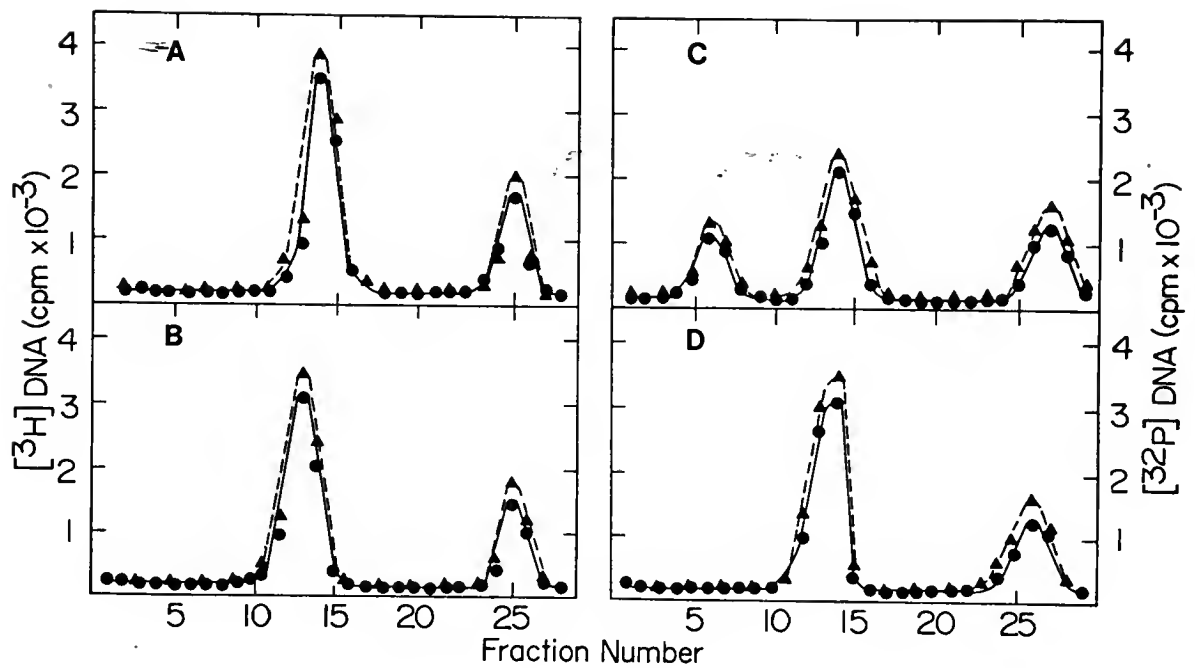

A
B
C
[3H] DNA (cpm x 10-3)
[32P] DNA (cpm x 10-3)
Fraction Number
5
10
15
20
25

ATP for 30 minutes af 37°C followed by incubation for 30 minutes with topoisomerase. A catenated pair of duplex molecules should collapse in alkali and sediment as a dimer, faster than denatured monomeric form I (West et al., 1982). When D-loop DNA was used, made by either thermal annealing or rec1 protein catalysis, a rapidly sedimenting peak of DNA containing both [^{3}H]- and [^{32}P]-labels was observed to form (Fig. 4-4). This population of molecules is probably two DNA duplex molecules linked together. The product of this reaction was characterized further and will be described below. Interestingly, in reactions where the topoisomerase was added with the rec1 protein and ATP, no dimer size DNA molecules were produced. This can be attributed to the fact that relaxation of form I DNA is fast and produces duplex molecules incapable of D-loop formation. These results indicate that the formation of D-loop molecules is a prerequisite for the catenation of two duplex molecules.

The cellular process of transcription naturally produces DNA molecules of three-stranded configurations. With increasing superhelicity, both the level of transcription and the stability of RNA-DNA hybrids (R-loops) are increased (Wang, 1974). RNA polymerase was added to reactions containing [^{3}H]- and [^{32}P]-labeled form I DNA of M13, rec1 protein and all four ribonucleotides, followed by addition of topoisomerase. The concerted action of three DNA enzymes produced dimer size DNA hybrid molecules containing both the [^{3}H]- and the [^{32}P]-labels (Fig. 4-4). Addition of 20 μM rifampicin completely eliminated the formation of joint molecules. The level of dimer sized molecules formed in part by the action of RNA polymerase was at a lower level than those achieved using D-loop DNA. This was probably due to a

Fig. 4-3. Linkage of form I DNA molecules promoted by homologous single stranded fragments.

Reaction mixtures of 200 µl containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP, 15 µM ^{3}H-labeled M13 form I DNA, 15 µM ^{32}P-labeled M13 form I DNA, 10 µM M13 single-stranded fragments, and 20 µg/ml rec1 protein were incubated at 37°C. After 20 minutes 200 units/ml topoisomerase was added and incubation continued for 20 minutes. Reactions were stopped and the DNA analyzed by centrifugation in alkaline sucrose gradients as in Figure 5. (A) minus fragments (B) topoisomerase added simultaneously with rec1 protein (C) complete mixture (D) G4 fragments added in place of M13 fragments. (●) ^{3}H-labeled DNA. (▲) ^{32}P-labeled DNA.


A
B
C
D
[3H] DNA (cpm x 10-3)
[32P] DNA (cpm x 10-3)
Fraction Number
5
10
15
20
25
1
2
3
4

contamination of endonuclease activity in the RNA polymerase (Fig. 4-4C, D). Taken together these results indicate that a three-stranded structure in either the D-loop or R-loop form can promote the homologous pairing of two duplex DNA molecules.

Characterization of Complexes Formed Between Intact Homologous Circular Duplexes

Based on the results obtained from the characterization of hemicatenanes formed between single stranded circles and superhelical duplex DNA, the possibility that fully catenated molecules were formed in the duplex-duplex reaction was unlikely. However, experiments were designed to directly test this possibility and to elucidate the structure formed in such a pairing reaction. [^{3}H]-labeled M13 Gori1, [^{32}P]-labeled G4 form I and G4 single stranded fragments were prepared. These substrates were reacted in a mixture containing recl protein, ATP and then topoisomerase. Dimer-sized product molecules were purified by centrifugation in a neutral sucrose gradient (Fig. 4-6A). To rule out the possibility that the duplexes were joined as full catenanes (Fig. 4-6D), the complex was cut at the unique BamHI site within the M13 Gori1 DNA or at the PstI site within the G4 DNA (Fig. 4-7). Because the DNA did not dissociate into full length linear molecules (Fig. 4-8, lanes g, h), the complex is not in the form of a full catenane. The change in mobility of the complex after restriction enzyme digestion is consistent with the pattern we would expect of a structure composed of two joined circular duplexes that had been cut in one duplex and then in the other.

Since it was now believed that the joint molecule made between two duplex DNA supercoils was in the form of a hemicatenane, experiments

Fig. 4-4. Linkage of form I DNA molecules promoted by RNA polymerase. Reaction mixtures of 200 µl containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP, 0.15 mM CTP, GTP, and UTP, 40 µM ^{3}H-labeled M13 form I DNA, 40 µM ^{32}P-labeled M13 form I DNA, 40 µg/ml rec1 protein and RNA polymerase were incubated at 37°C. After 20 minutes 200 units/ml topoisomerase was added and incubation continued for an additional 20 minutes. DNA was centrifuged in alkaline sucrose gradients as described in Figure 5. (A) No RNA polymerase (B) 0.5 units RNA polymerase, no rec1 protein (C) complete reaction, 0.5 units RNA polymerase (D) complete reaction, 1 unit RNA polymerase (E) complete reaction containing 1 unit RNA polymerase and 20 µM rifampicin (F) complete reaction containing 1 unit RNA polymerase minus UTP, CTP, GTP. (●) ^{3}H-labeled DNA. (▲) ^{32}P-labeled DNA.

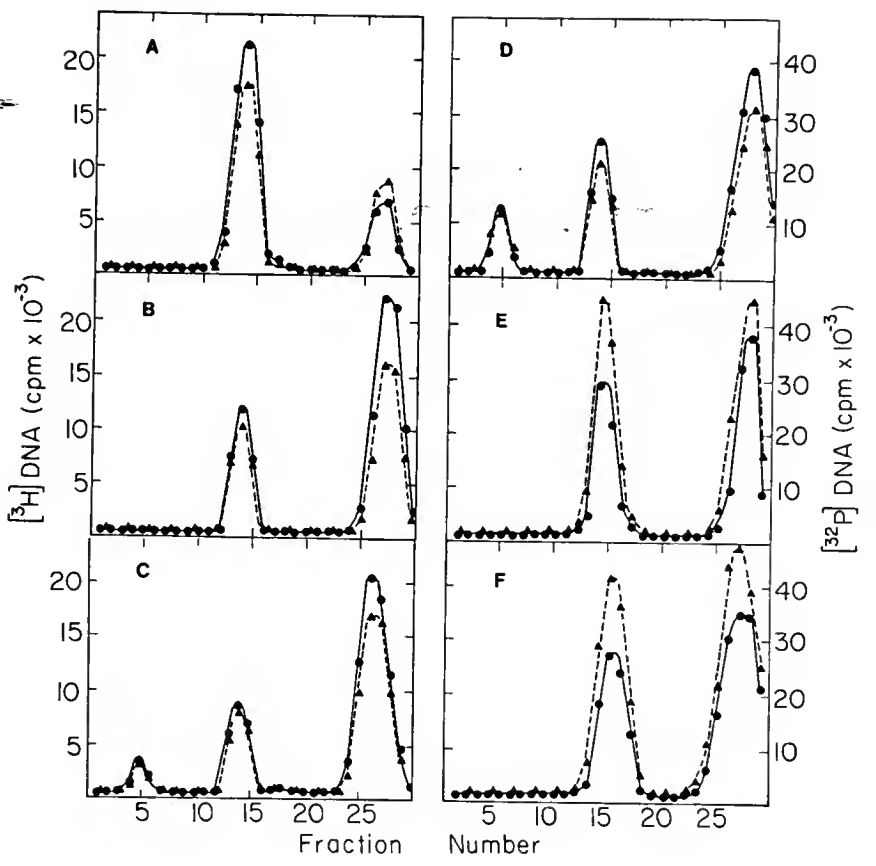

A
B
C
D
E
F
[3H] DNA (cpm x 10-3)
[32P] DNA (cpm x 10-3)
Fraction Number

Fig. 4-5. Analysis of product formed with homologous or heterologous combinations of form I DNA.

(A) Reaction mixture of 0.5 ml containing 35 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP, 200 μM ^3H-labeled M13 Gori1 form I DNA, 200 μM ^{32}P-labeled G4 form I DNA, 100 μM unlabeled G4 single stranded fragments, and 50 μg/ml rec1 protein was incubated at 37°C. After 20 minutes 300 units/ml topoisomerase was added and incubation continued for 20 minutes. The reaction was stopped by addition of 50 mM EDTA, the mixture layered on a neutral sucrose gradient in 1 M NaCl, and centrifuged at 25,000 rpm for 18 hours in a Beckman SW41 ti rotor. Fractions of 0.4 ml were collected and aliquots of 10 μl were removed for determining radioactivity. Fractions 11-14 containing the catenated product were pooled and the DNA was collected after precipitation with ethanol. (B) The reisolated catenated product was cleaved with BamHI in a reaction mixture of 100 μl containing 10 mM Tris pH 7.9, 6 mM $MgCl_2$, 150 mM NaCl, 30 μM DNA and 10 units/ml BamHI. After 2 hours at 37°C the DNA was analyzed by centrifugation in an alkaline sucrose gradient as in Figure 5. (C) ^{32}P-labeled G4 form I DNA and ^3H-labeled M13 Gori1 DNA converted to the linear form III by cleavage with BamHI were run as markers in an alkaline sucrose gradient in parallel with B. In D-F catenated product was prepared with a heterologous combination of form I DNAs. The reaction mixture of 200 μl containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM adenylyl-imidodiphosphate, 125 μM ^3H-labeled fd form I DNA, 125 μM ^{32}P-labeled G4 form I DNA, and 30 μg/ml rec1 protein was incubated at 37°C. After 20 minutes 250 units/ml topoisomerase was added and incubation continued for 20 minutes. The reaction was stopped by addition of EDTA and the product was isolated as in A. Reisolated catenated product was cleaved with restriction endonuclease PstI in a reaction mixture of 100 μl containing 10 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 50 mM NaCl, 2 μM DNA, and 2 units/ml PstI. After 2 hours at 30°C the mixture was divided into two equal portions. To one was added 50 mM EDTA and 1 M NaCl. After heating at 75°C for 10 minutes it was centrifuged in a neutral sucrose gradient containing 1 M NaCl for 18 hours at 25,000 rpm in the SW41 ti rotor. The other portion was denatured with alkali and centrifuged for 2 hours at 34,000 rpm. (D) Reisolated catenated product centrifuged in a neutral sucrose gradient 18 hours at 25,000 rpm in the SW41 ti rotor. (E) Product cleaved with PstI centrifuged in a neutral sucrose gradient. (F) Product cleaved with PstI centrifuged in an alkaline sucrose gradient. (●) ^3H-labeled DNA. (▲) ^{32}P-labeled DNA.

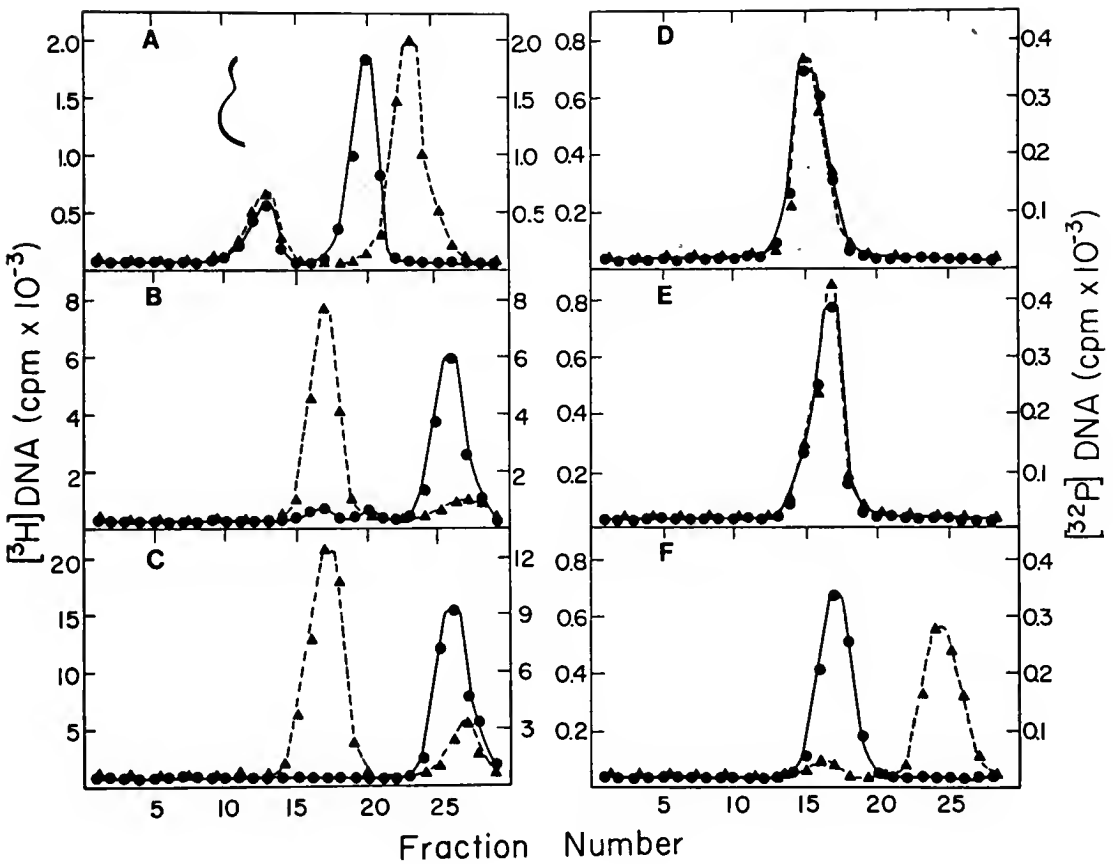

A
B
C
D
E
F
[3H]DNA (cpm x 10-3)
[32P] DNA (cpm x 10-3)
Fraction Number
5
10
15
20
25

Fig. 4-6. Linked pairs of circular DNA molecules.
(A) Fully catenated molecules (B) Hemicatenated molecules with multiple links (C) Hemicatenated molecules with a single link (D) Fully catenated molecules (E) Hemicatenated molecules with one pair of strands interlinked (F) Hemicatenated molecules with both pairs of strands interlinked (G) Hemicatenated molecules with a single link (H) Covalently linked molecules.

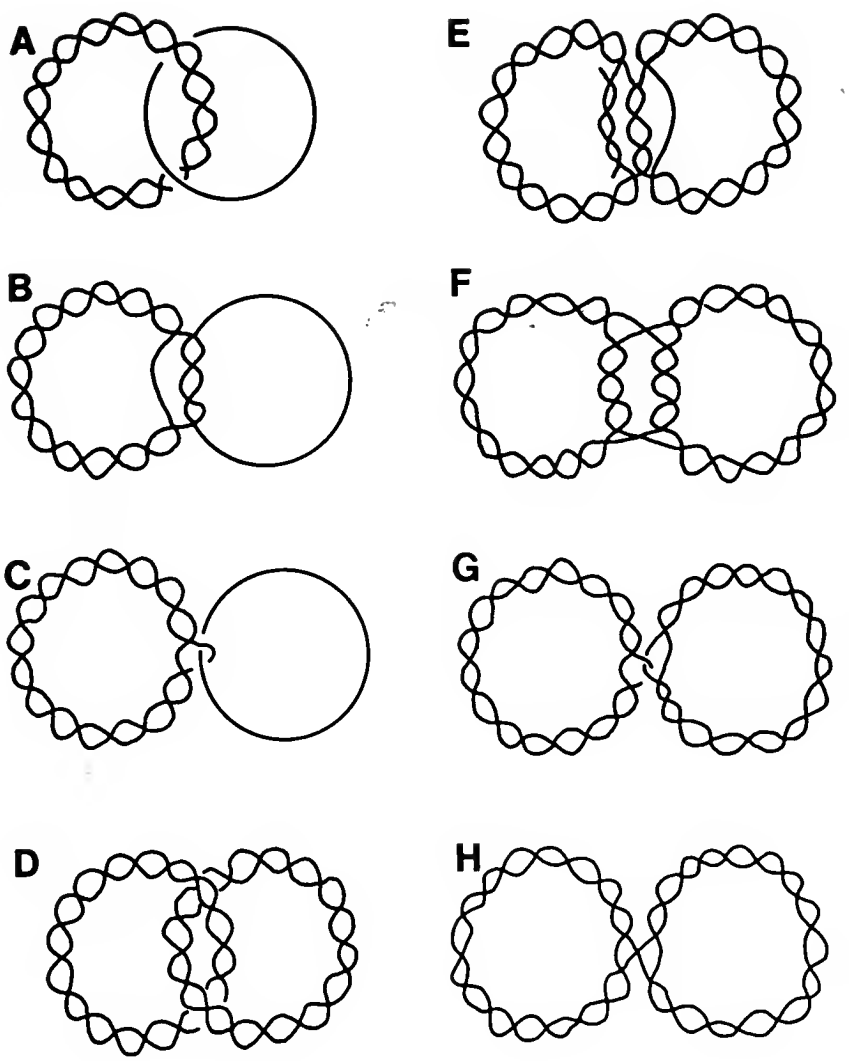

A
B
C
D
E
F
G
H

Fig. 4-7. Maps of DNA from phages M13 Goril, M13, fd, and G4.
The restriction sites shown have been taken from the data of Beck et al. (1978), Godson et al. (1978), Kaguni and Ray (1979), and van Wesenbeek et al. (1980).

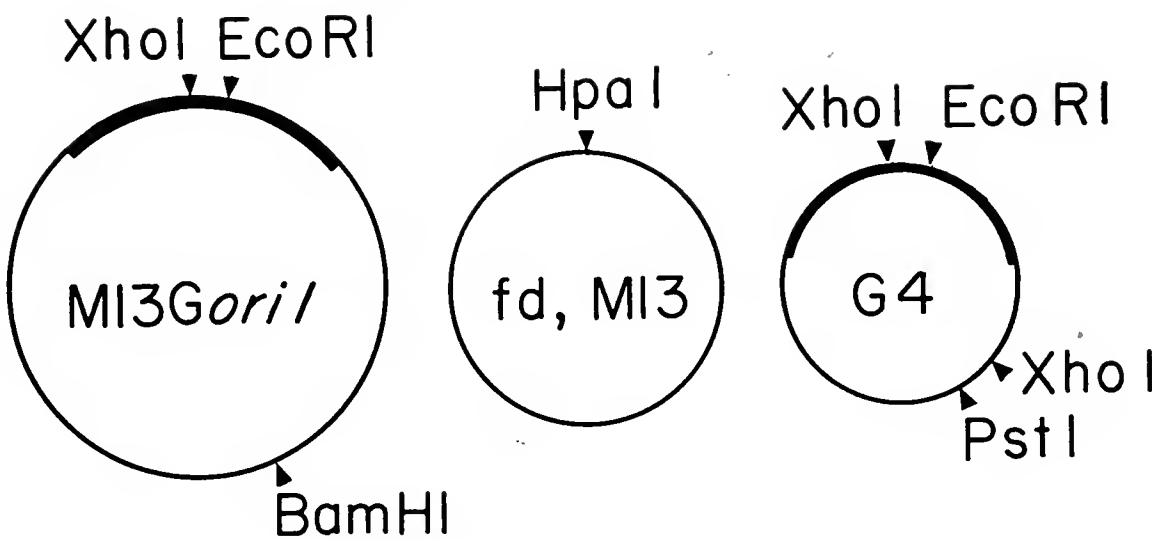
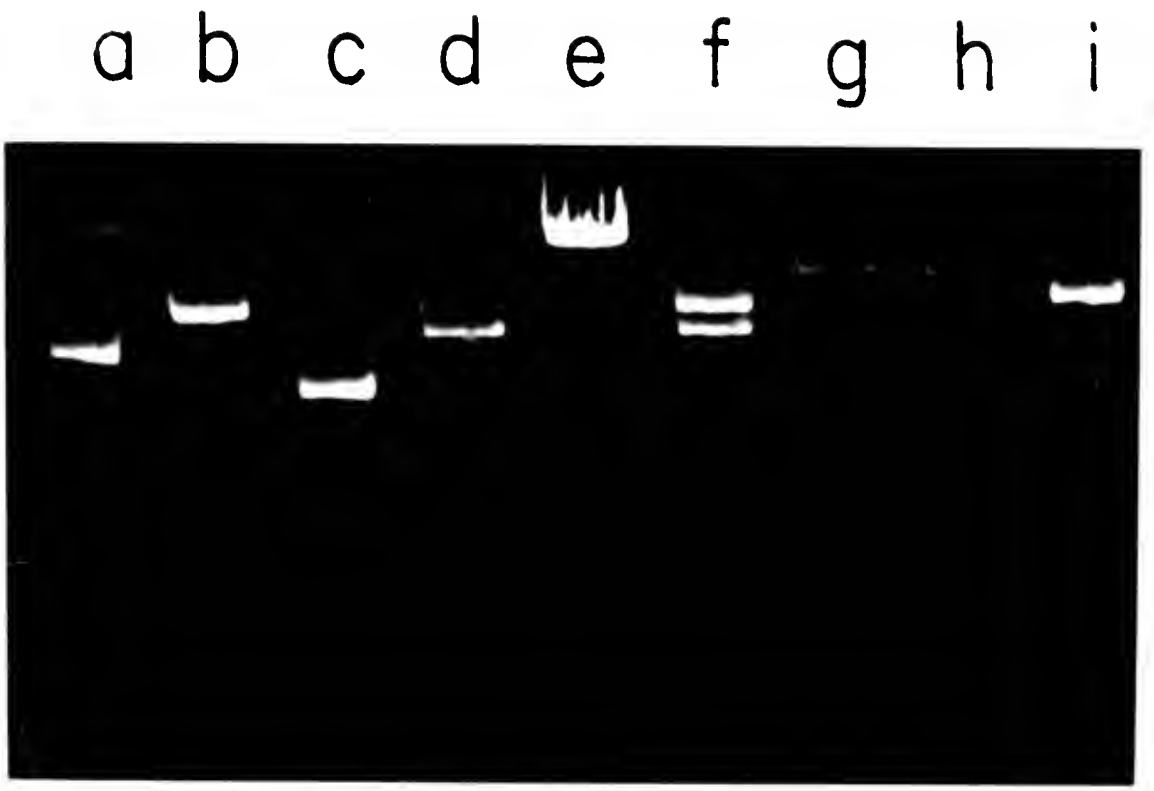

XhoI EcoRI
M13G*ori1*
BamHI
HpaI
fd, M13
XhoI EcoRI
G4
XhoI
PstI

Fig. 4-8. Analysis by agarose gel electrophoresis of the catenated product formed between homologous DNA duplexes.

The linked complex of M13 Gori1 form I DNA and G4 form I DNA was prepared as described in Figure 4-6A. Each lane was loaded with approximately 2.5 nmol of DNA. Electrophoresis was carried out as described in Appendix F. (A) M13 Gori1 form I DNA (B) M13 Gori1 DNA cut with EcoRI (C) G4 form I DNA (D) G4 DNA cut with EcoRI (E) reisolated catenated product (F) product cut with EcoRI (G) product cut with BamHI (H) product cut with PstI (I) product cut with BamHI and PstI.


a b c d e f g h i

were designed to test this hypothesis. Several structures were possible, all of which would be defined theoretically as hemicatenanes. The first (Fig. 4-6E) contains a region in which only one set of complementary strands from each parent duplex are interwound. An alternative structure (Fig. 4-6F) would be one in which both sets of compelementary strands are heteroduplexed. To look for the formation of singly or doubly interwound hemicatenanes, the joint molecules were made as described using M13 Gori1 and G4 form I DNA. The catenated molecules formed in this instance would be heteroduplexed only within the 2200 base pairs of the G4 section in M13 Gori1. A single EcoRI restriction site lies within this region in both substrates (Fig. 4-7). When the complex was cut with EcoRI, the molecules resolved into two full length linear molecules. Therefore, the hemicatenated dimer is likely to be a structure in which there are two sets of heteroduplexed strands (Fig. 4-7F).

There is an alternative explanation for the results obtained after digestion with EcoRI, depicted in Figure 4-6H, and referred to as a true Holliday structure. To eliminate this structure as a candidate for the complex, the joined molecules were cut with the restriction endonuclease BamHI and the products analyzed by centrifugation in gradients of alkaline sucrose. If the complex was in the Holliday structure, only linear and circular single strands would be liberated. The results, depicted in Figure 4-5 (B, C) reveal the liberation of G4 covalently closed duplex circles and full length linear strands. These results are consistent with the view that the complex is in the form of a double hemicatenane. Analysis of joined molecules of nonhomologous partners G4 and fd form I molecules, paired by recl protein in the

presence of adenylyl-imidodiphosphate, and toposiomerase revealed that this complex is a hemicatenane in which strands are linked but not base paired (Kmiec et al., 1983).

Discussion

Previous studies on the homologous pairing activity of the recl protein illustrated that superhelical duplexes and single stranded fragments, and linear duplexes and single stranded circles could be united in an efficient manner. The present studies add to the list of successful pairing partners, circular single stranded and circular duplex DNA molecules. The significance of these latest pairing reactions lies in the fact that neither substrate contains free ends. In addition; both combinations, single strand circle-duplex and duplex-duplex, require a single stranded stretch of DNA in a region of homology.

The recA protein of E. coli can pair duplex molecules if one of them contains a single stranded gap (Cunningham et al., 1980; West et al., 1981,1982). Using EM DasGupta et al. (1980) showed that single stranded DNA could be paired to its complement in a homologous duplex in the absence of free ends. Linkage of such molecules, using E. coli topoisomerase I, was demonstrated by Wu et al. (1983). These results coupled with the present observations strengthen the view that pairing can precede breakage during initiation of recombination.

In recl protein promoted pairing of homologous duplexes several reaction requirements must be met. Formation of the dimeric DNA complex, topologically linked, was dependent on the presnce of ATP, single stranded DNA and the order of enzyme addition. The role ATP plays in the pairing reaction is as yet unknown. One can speculate

that the energy required to unwind the superhelix may be derived from ATP hydrolysis in the presence of single stranded DNA. Since pairing reactions were unsuccessful in the absence of single stranded DNA, a cofactor in recl protein's ATPase activity, this hypothesis is attractive. When topoisomerase was added to reaction mixtures with the recl protein, no linked dimeric molecules were formed. This may be a reflection of the fact that fully relaxed covalently closed duplex molecules are not good substrates for the uptake of a single stranded fragment. These results implicate the energy of the superhelix itself in overall pairing reactions.

The governing role played by single stranded DNA in the initiation of recombination has been clearly illustrated (see Radding, 1982 for review). The present results reinforce this idea. Successful topological linkage of duplex DNA molecules relied heavily, not only on the presence of single stranded fragments, but also on the location of the three stranded region. It must be pointed out that no direct data has been presented which unequivocally shows that juxtaposition of the duplexes begins at a D-loop site. However, it is clear that this D-loop region is an essential part of the pairing pathway. Futhermore, heteroduplexed regions within dimeric molecules were present only at sites previously made three stranded. These results indicate that pairing of homologous sequences in the absence of a free end can occur; again suggesting that pairing before breakage is a viable mechanism in the initiation of recombination.

Although D-loop DNA is a well-known feature of mitochondrial DNA (Clayton, 1982), it is not difficult to envision three stranded structures occurring throughout the DNA chromosome. For example,

D-loops might form along linear chromosomes as a result of an uptake of redundant single strands during DNA synthesis (Lundquist and Olevera, 1982) or from short stretches of undirected replication. The transcriptional process can generate stable RNA-DNA hybrids (R-loops) and from the data presented here these R-loops can catalyze the formation of duplex-duplex complexes. Other laboratories have presented evidence, using λ phage, that chain elongation during transcription is important in homologous recA-independent recombination (Ikeda and Matsumoto, 1979). Furthermore, evidence has accumulated from studies of λ phage integration (Davies et al., 1972), mating-type switching (Klar et al., 1981) and rearrangements during immunoglobulin development (Van Ness et al., 1981) suggesting a relationship between transcription activities and site specific recombination.

Finally, Shibata et al. (1982) suggested that pairing along homologous regions of chromosomes aids in accurate segregation in meiosis. This naturally occurring activity may facilitate the creation of crossover junctions since the chromosomes are in homologous alignment. Once chromosomes are juxtaposed, the conjoining of the DNA duplexes to initiate recombination begins. The events which lead to the synapsis of DNA molecules are presently undefined.

CHAPTER FIVE
SYNPASIS OF DNA MOLECULES PROMOTED REC1 PROTEIN

The synapsis of homologous chromosomes prior to karyokinesis is a cellular event which assures a proper segration pattern. As early as 1911, Morgan theorized that such synapsed chromosomes could undergo genetic recombination. Until recently the forces bringing the DNA duplexes into homologous register have remained unknown and outside the realm of biochemistry. The pioneering work on the recA gene and its product have transformed cytogenetic observations into defined enzymological processes (for review see Radding, 1982). The eukaryotic rec1 protein, has been shown to carry out a variety of DNA pairing reactions crucial for initiation of recombination (Kmiec and Holloman, 1982, 1983, 1984; Kmiec et al., 1983). Pairing or synapsis of DNA molecules requires only an energy cofactor and sequence homology. It was previously established that homologous free ends are not a requirement (Kmiec et al., 1983) (see Chapter 4). It was clear, however, that stable heteroduplex molecules could be formed only in the presence of a homologous free end. Still a possibility existed that rec1 protein may be able to place two DNA molecules into homologous register regardless of the topological constraints.

In the absence of free ends, the recA protein can pair a single strand with its complement in a duplex molecule promoting the formation of a different kind of joint in which the strands are paired, but not interwound (Bianchi et al., 1983). This so-called paranemic joint is

unstable and has a linking number of zero. In contrast, the plectonemic joint in which the DNA strands are interwound has a linking number of non-zero and is stable.

Experiments were designed to explore the side-by-side pairing of DNA molecules promoted by the rec1 protein in an assay which measured the initiation complex consisting of the two DNA molecules and the enzyme. This ternary complex may closely resemble the molecular framework present when rec1 protein brings the molecules into homologous alignment. Information gained from this study will contribute to the understanding of rec1-protein promoted DNA synapsis, the initial step in strand transfer reactions.

Formation of Stable Complexes

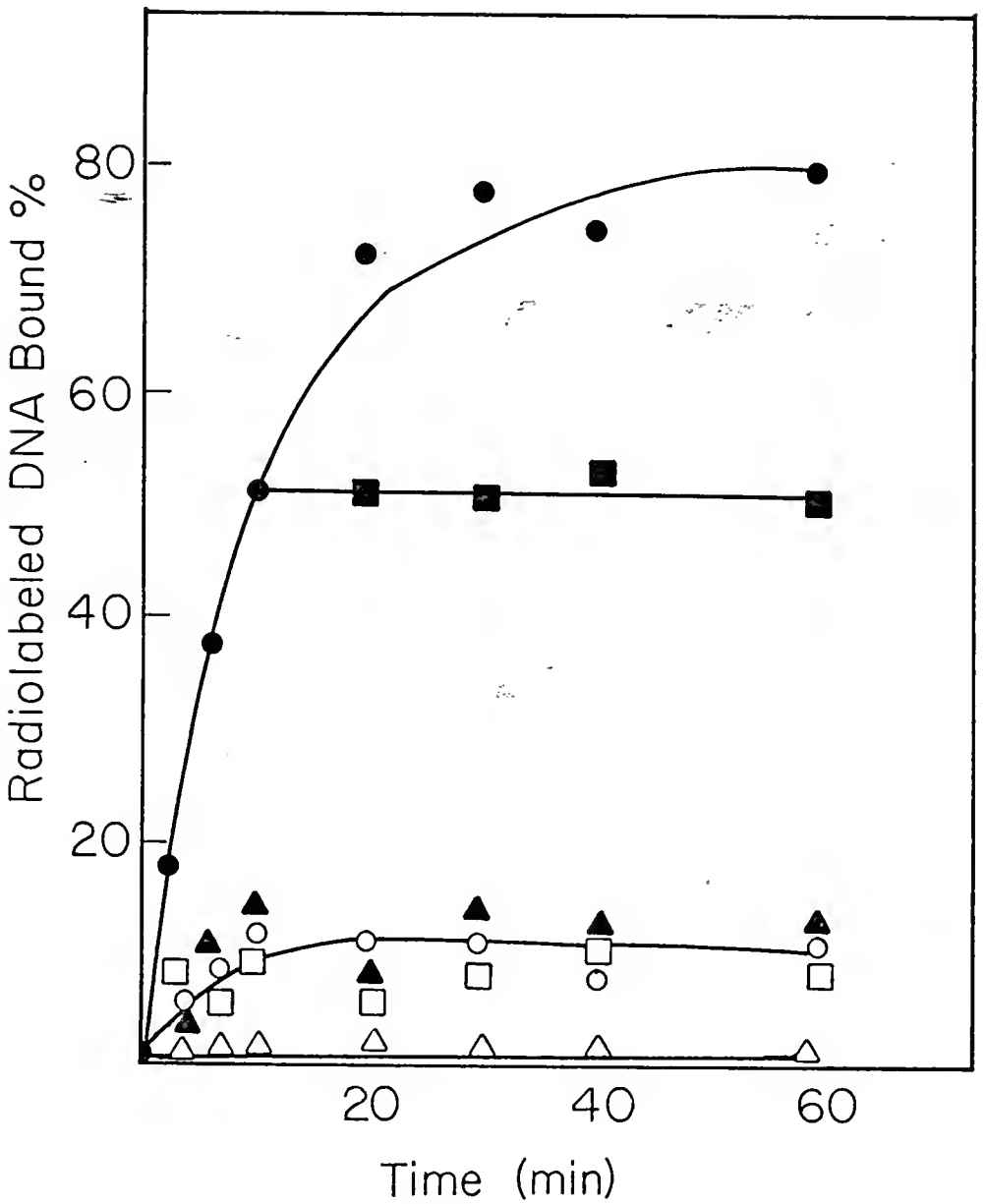
It had previously been observed that rec1 protein could pair DNA molecules lacking free ends in preparation for topological linkage by Ustilago topoisomerase (Chapter 4). The initial event of the synapsis reaction was reasoned to be an interaction between rec1 protein and the two DNA molecules. To study the formation of synaptic ternary complexes, a nitrocellulose assay was used similar to the one developed in studies on lac repressor (Riggs et al., 1968). This assay monitors the formation of protein-DNA complexes because only labeled DNA bound by protein is retained on the nitrocellulose filters. In washings of low ionic strength buffer, DNA itself is not retained. Calf thymus H-1 histone was used to measure the efficiency and accuracy of the assay. When 0.1 µg of H-1 histone was incubated under standard reaction conditions with labeled duplex linear DNA, approximately 80% of the DNA was retained by the filter (data not shown).

When recl protein was added separately to binding reactions, linear duplex DNA was bound to a low level, but single stranded DNA was retained to a much higher level. However, duplex linear DNA could be retained at high levels when homologous single stranded DNA was included in the reaction mixture (Fig. 5-1). The stimulated binding of duplex was dependent on ATP. Treatment with proteinase K dissociated complexes already formed.

When recl protein was reacted with single stranded DNA and homologous linear duplex in the presence of ADP, the formation of complexes was inhibited, confirming an earlier observation in which ADP was found to inhibit heteroduplex formation early in the reaction.

The type of linear duplex DNA used up to this point has been completely homologous to the single stranded circle and therefore could facilitate the formation of stable heteroduplexes. These joint molecules have been found to be stable under a variety of conditions including the removal of protein molecules (Kmiec and Holloman, 1983). Therefore, to study synapsis separately from heteroduplex formation and to avoid complications arising after recl protein completed synapsis and began to drive strand exchange, DNA molecules which would be topologically barred from forming authentic Watson-Crick base pairs were employed. By cutting the chimeric phage M13 Gori1 with the restriction endonuclease XhoI, a duplex linear molecule is created which contains M13 sequences internally flanked by nonhomologous G4 stretches (Kaguni and Ray, 1979; Godson et al., 1978). In reaction mixtures where M13 single stranded DNA was used, pairing could only occur at internal sites without the benefit of a homologous free end. When recl protein was added to a reaction containing these two DNA

Fig. 5-1. Nitrocellulose filter assay for synaptic complexes.
A. Reactions (500 µl) contained 7.5 µM M13 single-stranded circular DNA, 15 µM ^{3}H-labeled M13 linear duplex DNA, 1 mM ATP and 15 µg/ml rec1 protein (●). Aliquots (50 µl) were removed at the indicated times, brought to 25 mM EDTA, and washed onto nitrocellulose filters and radioactivity determined. In one reaction the aliquots were treated with treated with proteinase K (100 g/ml) for 10 minutes before filtering through nitrocellulose (△). In another reaction single stranded M13 DNA was omitted (▲) or replaced by single stranded ϕX174 DNA (○). In other reactions 2 mM ADP was added either initially (□) or at the time indicated by the arrow (■).


Radiolabeled DNA Bound %
80
60
40
20
20
40
60
Time (min)

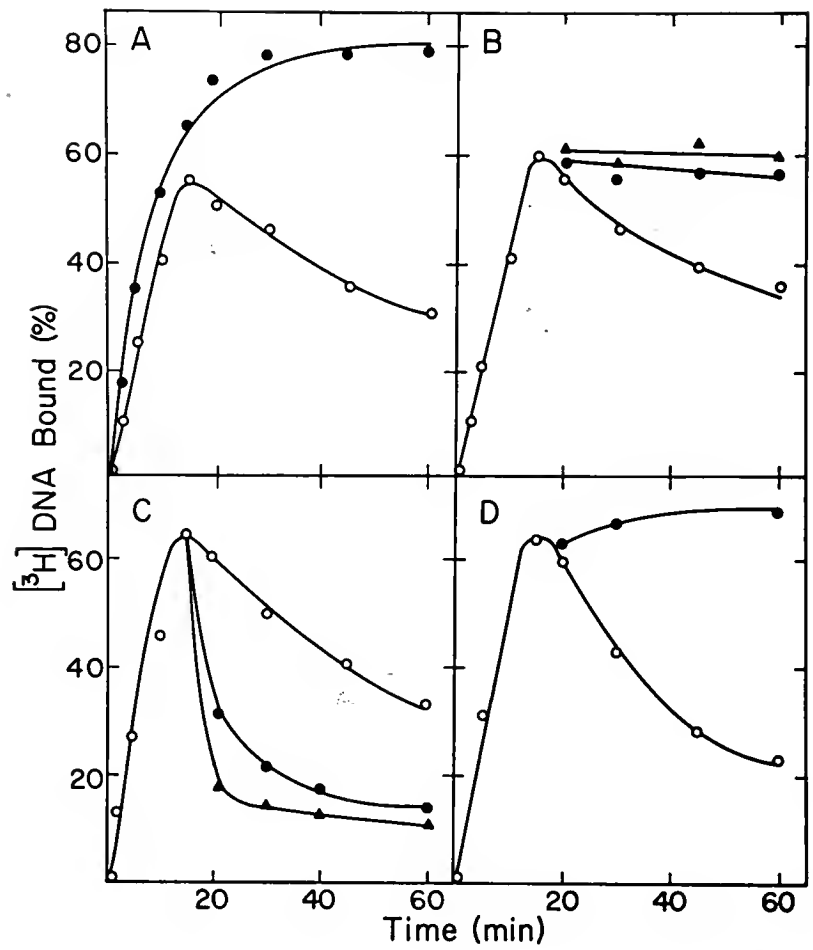
molecules and ATP, complexes formed rapidly (Fig. 5-2). However, after 20 minutes, the level of complexes decreased steadily. In sharp contrast totally homologous M13 duplex linear DNA and M13 single stranded circles formed complexes with rec1 protein, but continued rising to a plateau. When ADP was added to completely homologous complexes it stopped further complex formation but those already formed remained stable (Fig. 5-1). ADP rapidly dissociated complexes that contained molecules paired in the absence of free ends (Fig. 5-2C). Removal of ADP by regeneration back to ATP (see Appendix E) increased the level of complex formation (Fig. 5-2D). Dissociation of complexes could be prevented by adding topoisomerase or adenylyl-imidodiphosphate (Fig. 5-2B). Stability of synaptic complexes appeared to be greatly enhanced when true Watson-Crick base pairs were allowed to form. Paranemic joints, conjoined molecules lacking Watson-Crick base pairing, are less stable than their plectonemic counterparts (Bianchi et al., 1983). These data reinforce that observation and extend it by implicating ADP in the dissociation process.

The size of the paranemic joint formed between circular single stranded DNA and linear duplex was measured by a simple nuclease digestion assay. These experiments showed that the formation of joint molecules occurring in the middle of the duplex is accompanied by complete unwinding of the homologous region (Kmiec and Holloman, 1984). Furthermore, these same sutdies indicated that ADP prevented the unwinding of duplex DNA.

Paranemic Joints Contain Left-Handed Z-DNA

An array of molecular frameworkd could be envisioned for the structure of the paranemic joint. First, the two complementary

Fig. 5-2. Paranemic joint molecules are dissociated by ADP.
A. Reaction mixtures (0.45 ml) containing either 15 μM ^{3}H-labeled linear duplex M13 DNA (●) or M13 Gori1 DNA (○), 7.5 μM single-stranded circular M13 DNA, 1 mM ATP, and 15 μg/ml rec1 protein were incubated at 37°C. Radioactive DNA retained by nitrocellulose filters was determined from aliquots removed at the indicated times.
B. Reaction mixture (0.85 ml) containing 15 μM ^{3}H-labeled linear duplex M13Gori1 DNA, 7.5 μM single stranded circular M13 DNA, 1 mM ATP, and 15 μg/ml rec1 protein was incubated at 37°C. After 15 minutes the reaction was split into portions. To one was added 2 mM adenylyl-imidodiphosphate, and to another was added 200 units/ml topoisomerase. Aliquots were removed at the indicated times and radioactivity retained by nitrocellulose was determined. No addition (○); topoisomerase added (▲); adenylyl-imidodiphosphate added (●). C. To a reaction identical to that in B was added ADP. Control (○); 0.5 mM ADP (▲); 2 mM ADP (●). D. To a reaction identical to that in B was added 10 mM creatine phosphate and 20 units/ml creatine phosphokinase. Control (○); plus ATP regenerating system (●).


A
B
C
D
80
60
40
20
60
40
20
20
40
60
20
40
60
[3H] DNA Bound (%)
Time (min)

strands, one from the circle, the other from the duplex, could lay side-by-side. Second, normal right-handed helices could form although this formation would be difficult topologically. Finally, one might predict the structure of DNA in a paranemic joint to be one of alternating stretches of right-handed and left-handed DNA. To detect formation of left-handed sgements in paranemic joint molecules antibodies for left-handed Z-DNA were used (Lafer et al., 1981). The experimental procedure was to coat wells in microtiter dishes with Z-DNA antibody and then measure how much labeled duplex DNA was bound in the wells after reacting with single stranded DNA and rec1 protein to form a paranemic joint. As a control, to ensure the antibody was specific, the retention of authentic Z-DNA compared to the B-form of the same DNA was measured. Z-DNA was made by brominating poly(dG-dC) and following the inversion of the circular dichroism spectrum to monitor trasnsition to the left-handed form (Lafer et al., 1981). The brominated and untreated polymers were then radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and the efficiency that the B and Z forms were bound by the Z-DNA antibody was compared. Under comparable conditions less than 4% of the B form was bound when 75% of the Z-form was bound. When antibody from normal serum was tested there was no specificity for Z-DNA. In an additional control using labeled single-stranded M13 DNA, only a low level of nonspecific binding to antibody, about 12%, the same level of DNA bound to antibody from normal serum.

Having established the specificity of the Z-DNA antibody, it was used as a probe to monitor formation of left-handed DNA during synapsis. Reaction mixtures of rec1 protein, single stranded circular

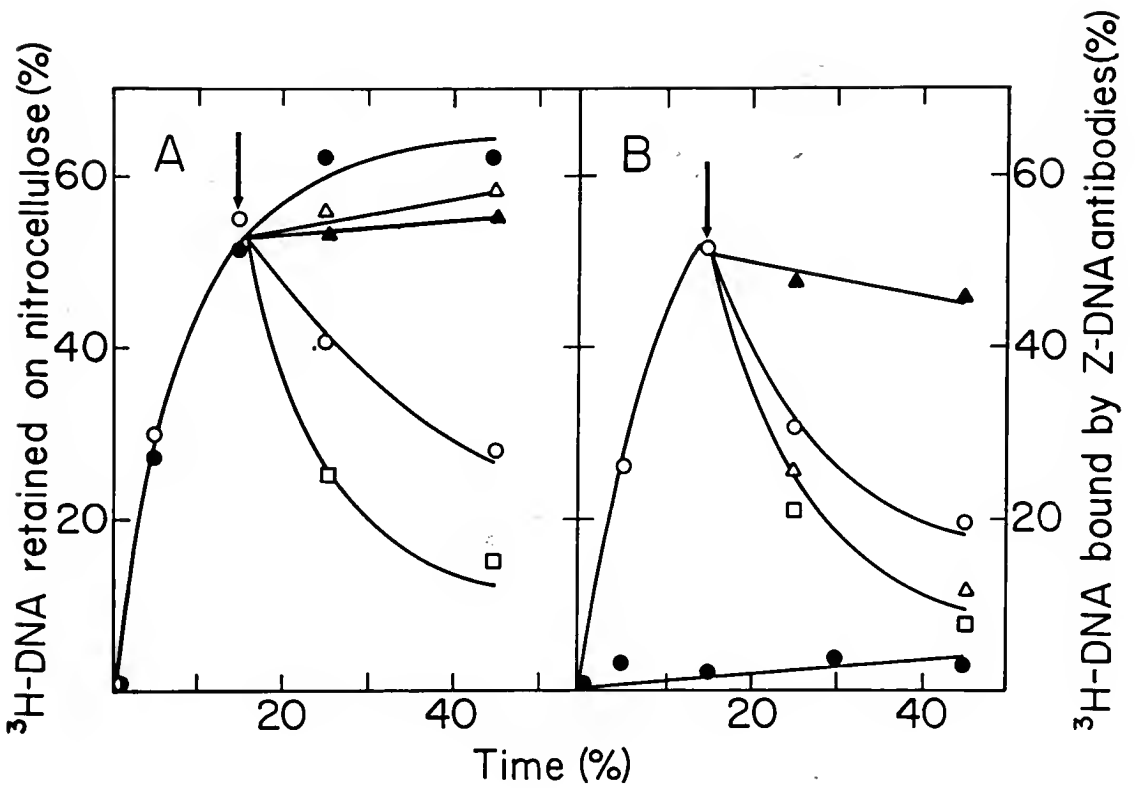
M13 DNA and ^{3}H-labeled linear duplex M13 DNA, showed that a high level of ternary complexes was formed as measured by the nitrocellulose filter assay (Fig. 5-3A), but little ^{3}H-labeled DNA was bound by Z-DNA antibody (Fig. 5-3B). In this case the heteroduplex joint formed would have the interwound or plectonemic structure of B-form DNA. When the M13 linear duplex DNA was replaced with M13 Gori1 DNA the picture was quite different. The rate of formation of synaptic complexes was accompanied by the parallel formation of left-handed DNA. The kinetics of appearance of both Z-DNA and synaptic complexes were almost identical. Binding of ^{3}H-labeled M13 Gori1 DNA by Z-DNA antibody rose to a maximum then decreased rapidly. Addition of ADP increased the rate of dissociation of the DNA from the antibody complex. Removing ADP from the reaction by regenerating it back to ATP stabilized the DNA structure bound by the Z-DNA antibody, but adding topoisomerase to catalyze interwinding of DNA strands eliminated the high level of binding. These observations suggest that the paranemic heteroduplex joint formed in reaction promoted by rec1 protein contained DNA at least partly in the Z form.

Discussion

The data obtained from the present studies suggest that the initiation of strand transfer reactions promoted by rec1 protein can occur in the absence of a free end. The synapsis of the DNA molecules is dependent only on homology and ATP. However, to form a stable heteroduplex a free end in one of the synapsed molecules must be available. This stability is reflected by the sensitivity to ADP demonstrated by paranemic joints but not by plectonemic joints. Furthermore, this data suggest that single stranded circular DNA and

Fig. 5-3. Paranemic joints contain left-handed DNA.

Reactions containing ^{3}H-labeled linear duplex M13 Gori1 DNA, single-stranded circular DNA, ATP, and rec1 protein were carried out as in the legend to Figure 5. After incubation for 15 minutes, portions were mixed with 2 mM ADP, 10 mM creatine phosphate plus 20 units/ml creatine phosphokinase with or without 200 units/ml topoisomerase. In another control completely homologous ^{3}H-labeled linear duplex M13 DNA was substituted for M13Gori1 duplex DNA. At the indicated times two sets of aliquots were removed. ^{3}H-labeled DNA retained by nitrocellulose was measured in one set, and DNA bound by Z-DNA antibody was measured in the other set. Control plectonemic reaction containing ^{3}H-labeled linear duplex M13 DNA (●); paranemic reaction containing ^{3}H-labeled linear duplex M13Gori1 DNA (○); ADP added (□); creatine phosphate plus creatine phosphokinase (△); creatine phosphate plus creatine phosphokinase, followed by topoisomerase (▲).


A
B
60
40
20
20
40
Time (%)
^{3}H-DNA retained on nitrocellulose (%)
^{3}H-DNA bound by Z-DNA antibodies (%)

linear duplex DNA can be paired in a side-by-side fashion if stretches of left- and right-handed helices are allowed to form. Evidence that the recA protein can recognize homology and promote side-by-side pairing of the same substrates was first obtained by DasGupta et al. (1980) using the electron microscope. When recA protein pairs these types of DNA molecules, the duplex becomes extensively unwound (Wu et al., 1983). The formation of paranemic joints was found to be sensitive to ADP and that continued hydrolysis of ATP by recA protein was essential for the integrity of paranemic joints (Wu et al., 1982; Bianchi et al., 1983).

The results obtained from studies of recA protein- and rec1 protein-promoted paranemic joint formation have led to similar conclusions. However, rec1 protein has been found to be under a more restrictive control of ADP. When rec1 protein brings together a single stranded DNA molecule and a homologous duplex, the stability of the nascent paranemic joint formed is controlled by ADP. Complexes are rapidly dissociated by low levels of ADP. After isomerization and conversion from a paranemic to plectonemic joint, ADP no longer has an effect. ADP may control the initiation of recombination by destabilizing the paranemic joint.

Reaction of Z-DNA antibody with the nascent heteroduplex structure indicates that regions of left-handed Z-DNA are formed during the development of a paranemic joint. This configuation of alternating left- and right-handed helices alleviates some of the topological constraints present at the paranemic site. Such a structure has been proposed for form V DNA (Pohl et al., 1982) which arises from annealing

complementary single stranded circular DNA rings (Stettler et al., 1979). As expected, Z-DNA antibodies interact with form V DNA.

The Z-DNA conformation may play a regulatory role in biological systems, although little direct data is currently available. Drosophila polytene chromosomes were demonstrated to contain regions of Z-DNA (Nordheim et al., 1981). The macronucleus of the ciliated protozoan, Stylonichia, and certain plant nuclei were found to contain DNA in the Z-form (Lipps et al., 1983). However, the development of a paranemic joint between two DNA molecules promoted by rec1 protein is the first evidence for the formation of Z-DNA as a result of a molecular process.

CHAPTER SIX
HYSTERIC REGULATION OF REC1 PROTEIN PRIOR TO DNA SYNAPSIS

The molecular processes leading to formation of the heteroduplex joint have been envisioned as a series of steps whereby homologous DNA molecules are brought into register and strand exchange begins. Formation of the heteroduplex joint can be catalyzed *in vitro* by the *Escherichia coli* recA protein. Several steps have been distinguished in the reactions. Prior to the actual exchange of strands and growth of the heteroduplex joint, recA protein first slowly polymerizes on single stranded DNA then promotes synapsis, the pairing of homologous DNA molecules. The presynaptic polymerization step is important for the following synaptic step of conjunction and alignment.

ADP inhibits formation of the heteroduplex joint catalyzed by recA protein. The sensitive phase in the overall reaction is the presynaptic step. ADP promotes dissociation of the complexes of recA protein and single stranded DNA. DNA dependent ATPase activity associated with recA protein generates enough ADP through hydrolysis to bring about dissociation. Addition of *E. coli* single stranded DNA binding protein stabilizes the recA protein-DNA complex by raising the threshold level of the ADP/ATP ratio necessary for dissociation.

The rec1 protein of Ustilago also promotes synapsis and strand exchange of homologous DNA molecules. As with *E. coli* recA protein, ADP inhibits homologous pairing catalyzed by Ustilago rec1 protein.

ADP was shown to promote dissociation of nascent synaptic complexes but complexes could be regenerated upon removal of ADP. The kinetics of this reversal are anomalous, however.

The nature of this dissociation is as yet unknown. ADP appears to play an important role in controlling synapsis in rec1 protein-promoted pairing reactions. The experiments described in this section were designed to elucidate the mechanism by which ADP modulates synapsis. The results of such experiments outline the presynaptic stage of strand transfer reactions and the molecular components which control it.

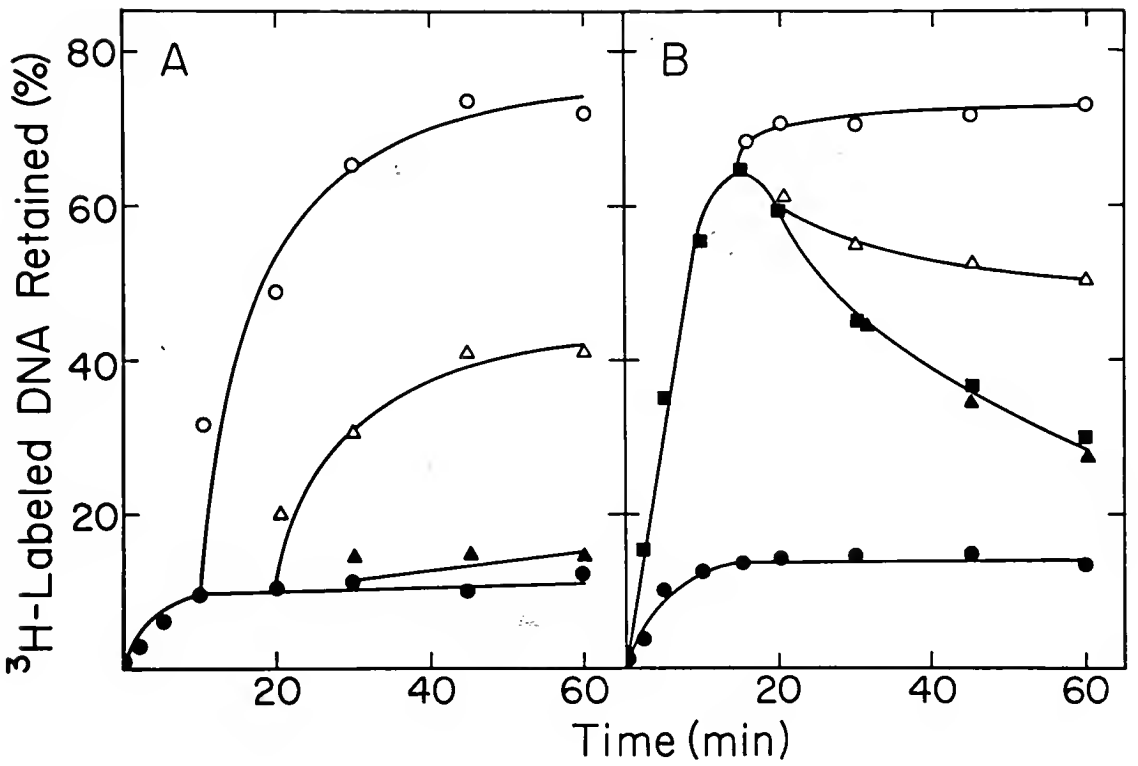
Kinetics of Synapsis are Anomalous

The rec1 protein can pair circular single stranded DNA and linear duplex in a reaction where homologous free ends are lacking. The complex thus created is known as a paranemic joint, contains stretches of Z-DNA and is sensitive to dissociation by ADP (see Chapter 5). The dissociation of paranemic joint molecules was stopped and their reformation stimulated when the reaction was coupled with creatine phosphate and creatine phosphokinase to regenerate ATP from ADP. However, the kinetics of this reversal were anomalous. Regeneration of ATP halted the decay of paranemic joints, but only within a narrow window of time (Fig. 6-1B). A similar anomaly was evidence in the kinetics of synapsis of circular single strands with totally homologous linear duplex molecules. Rapid formation of synaptic complexes occurred once ADP present initially in the reaction was regenerated to ATP. But the rate and extent of formation of synaptic complexes fell to almost zero if the regeneration system was added to the reaction beyond a certain time. A number of controls demonstrated that rec1 protein was by

Fig. 6-1. Anomalous kinetics of synapsis.

A. Reaction mixture containing 7.5 μM single-stranded circular M13 DNA, 15 μM ^{3}H-labeled M13 linear duplex DNA, rec1 protein and 1 mM ADP was incubated at 37°C. At the indicated times the reaction was split into portions. To added portion was removed and creatine phosphate and creatine phosphokinase to regenerate ADP to ATP. The level of synaptic complexes was determined from small aliquots. Control with no ATP regenerated (●); ATP regenerated at 10 minutes (○); 20 minutes (△); 30 minutes (▲).

B. Reaction mixture containing single-stranded circular M13 DNA, ^{3}H-labeled M13 Gori1 linear duplex DNA, rec1 protein and 1 mM ATP was incubated at 37°C. At the indicated times the ATP regenerating system was added. Control, with no ATP regeneration system added (■); ATP regenerated at 15 minutes (○); 20 minutes (△); 30 minutes (▲); control with 1 mM ADP substituting for ATP, no regeneration (●).


A
B
80
60
40
20
20
40
60
20
40
60
^{3}H-Labeled DNA Retained (%)
Time (min)

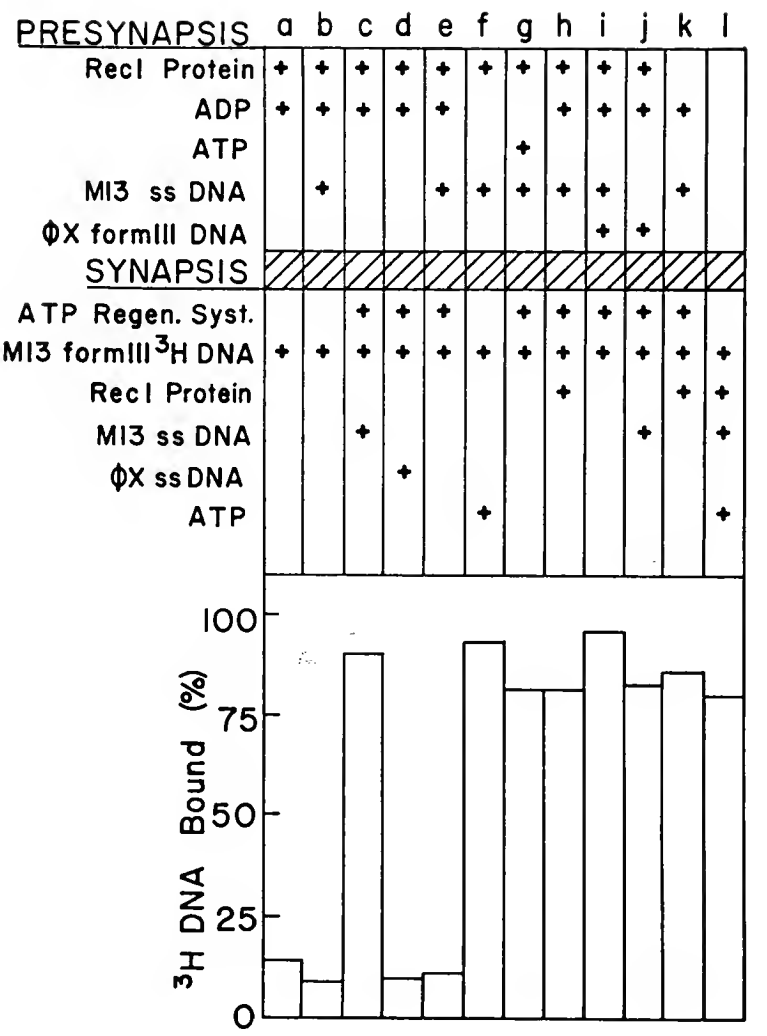
itself stable to the moderate warmth of the reaction temperature over the course of time (e.g., Fig. 6-2F).

Inactivation of Rec1 Protein During Presynapsis

Synapsis must comprise several phases since the overall process presumably involves recognition, conjunction and alignment of homologous DNA sequences. Synapsis was measured using a nitrocellulose filter assay to monitor formation of the synaptic product of circular single strands and radiolabeled homologous linear duplex DNA paired together by rec1 protein. To understand the synapsis reaction more fully, the experimental design was changed to allow greater flexibility in examining the action of individual components. A homologous combination of circular single stranded DNA and labeled linear duplex was used but the reaction was broken down into two steps, ordering the addition of components. In the first step, rec1 protein was preincubated with the circular single stranded DNA. In the second step, a source of ATP and the labeled linear duplex DNA was supplied to complete the homology requirement. The second step was referred to as synapsis, because here all components necessary for homologous pairing were present. The first was called presynapsis because at least one of the homologous DNA partners was held back.

When rec1 protein and ADP were preincubated for 20 minutes at 37°C, then unlabeled circular single stranded M13 DNA and ^{3}H-labeled linear duplex M13 DNA added, with regeneration of ADP to ATP, there was efficient production of synaptic complexes as measured by a big increase in retention of labeled DNA on nitrocellulose filters (Fig. 6-2C). Thus, rec1 protein remains active when preincubated under these conditions. Only a low level of duplex DNA was retained when

Fig. 6-2. Inactivation of rec1 protein during a presynaptic step.
Reactions (50 µl) containing, in various combinations as indicated, 15 µg/ml rec1 protein, 1 mM ADP, 1 mM ATP, 7.5 µM M13 single-stranded circular DNA, and 15 µM ΦX174 linear duplex DNA (form III) were incubated at 37°C. After 20 minutes ATP was regenerated as indicated by addition of creatine phosphate and creatine phosphokinase and other components were added including 15 µM ^{3}H-labeled M13 linear duplex DNA, 15 µg/ml rec1 protein 7.5 µM single-stranded circular DNA of M13 or ΦX174, and 1 mM ATP. Reaction continued for 30 minutes and the level of synaptic complexes was determined.


PRESYNAPSIS
a b c d e f g h i j k l
RecI Protein
ADP
ATP
MI3 ss DNA
ΦX formIII DNA
SYNAPSIS
ATP Regen. Syst.
MI3 formIII ³H DNA
RecI Protein
MI3 ss DNA
ΦX ss DNA
ATP
³H DNA Bound (%)
100
75
50
25
0

single stranded M13 DNA was left out (Fig. 6-2A), when nonhomologous ϕX174 DNA was substituted (Fig. 6-2D), or when no ATP was regenerated (Fig. 6-2B). Surprisingly, formation of synaptic complexes was totally inhibited when rec1 protein was preincubated with both ADP and single stranded DNA (Fig. 6-2E). Inhibition of synapsis was directly related to the concentration of ADP in the presynaptic step (Fig. 6-3). No inhibition was observed when ADP was deleted or when ATP was used in place (Fig. 6-2F, G). ADP did not inhibit the rate or level of binding of rec1 protein to single stranded DNA, nor promote dissociation of the protein from single stranded DNA.

When 76% of labeled input single stranded DNA was retainable on a nitrocellulose filter through binding with rec1 protein, the t1/2 of dissociation, as measured by release of labeled DNA from the filter, was >60 minutes after addition of a 10-fold excess of unlabeled single stranded DNA with or without ADP. The explanation for inhibition appeared to be more complicated, related somehow to interaction of rec1 protein with both single stranded DNA and ADP. Indeed when rec1 protein was preincubated with single stranded DNA and ADP, we found that severity of inhibition at synapsis was a function of the time of presynapsis. Thus it seems likely that the anomalous time dependent decay in active formation of synaptic complexes is a consequence of this inhibition.

Presynaptic Inhibition can be Blocked

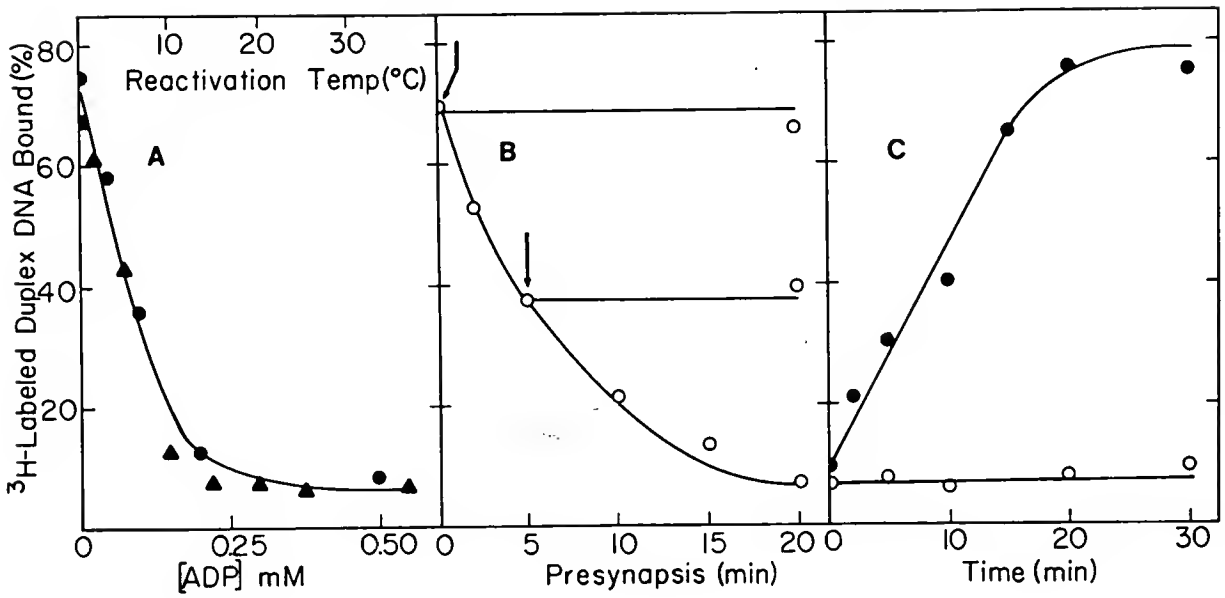
Inhibition of synapsis brought about by preincubating rec1 protein with ADP and single-stranded DNA can be blocked. Nonhomologous linear duplex DNA added along with rec1 protein, single-stranded DNA, and ADP in the preincubation mixture prevented inhibition at synapsis

Fig. 6-3. Parameters of presynaptic inhibition.

A. Reactions were carried out stepwise as described in Appendix F. In the first step the reaction mixture contained single-stranded circular M13 DNA, rec1 protein and various concentrations of ADP as indicated (●). After 20 minutes, the ATP regenerating system was added along with ^{3}H-labeled M13 linear duplex DNA and synaptic complexes were measured after a further incubation of 30 minutes. In a second reaction, the mixture was preincubated at 37°C for 20 minutes, then held at the indicated temperatures (▲) for 15 minutes before the final incubation at 37°C with ^{3}H-labeled M13 linear duplex DNA, creatine phosphate and creatine phosphokinase.

B. Reaction mixture containing rec1 protein, single-stranded circular M13 DNA and ADP was preincubated at 37°C for increasing lengths of time up to 20 minutes. Aliquots were removed at various times and the ATP regenerating system and ^{3}H-labeled M13 linear duplex DNA were added. synaptic complexes were measured 30 minutes later. In separate reactions ϕX174 linear duplex DNA was included in the mixture during preincubation either from the start for a total of 20 minutes or added 5 minutes after preincubation had begun for a total of 15 minutes (indicated by the arrows.)

C. After preincubating reactions containing rec1 protein, ADP, and single-stranded circular M13 DNA for 20 minutes at 37°C, the mixtures were placed at 0°C (●) or kept at 37°C (○) for the indicated times before adding ^{3}H-labeled M13 linear duplex DNA and regenerating ATP.


^{3}H-Labeled Duplex DNA Bound(%)
80
60
40
20
10
20
30
Reactivation Temp(°C)
A
0
0.25
0.50
[ADP] mM
B
0
5
10
15
20
Presynapsis (min)
C
0
10
20
30
Time (min)

(Fig. 6-2I, Fig. 6-3). In other words, formation of synaptic complexes with a homologous combination of single and double stranded molecules was not poisoned so long as a nonhomologous duplex was present. The duplex DNA acts as an effector to prevent inhibition rather than to reverse it. Rec1 protein poisoned by ADP and single stranded DNA could not be reactivated by addition of nonhomologous duplex (Fig. 6-3).

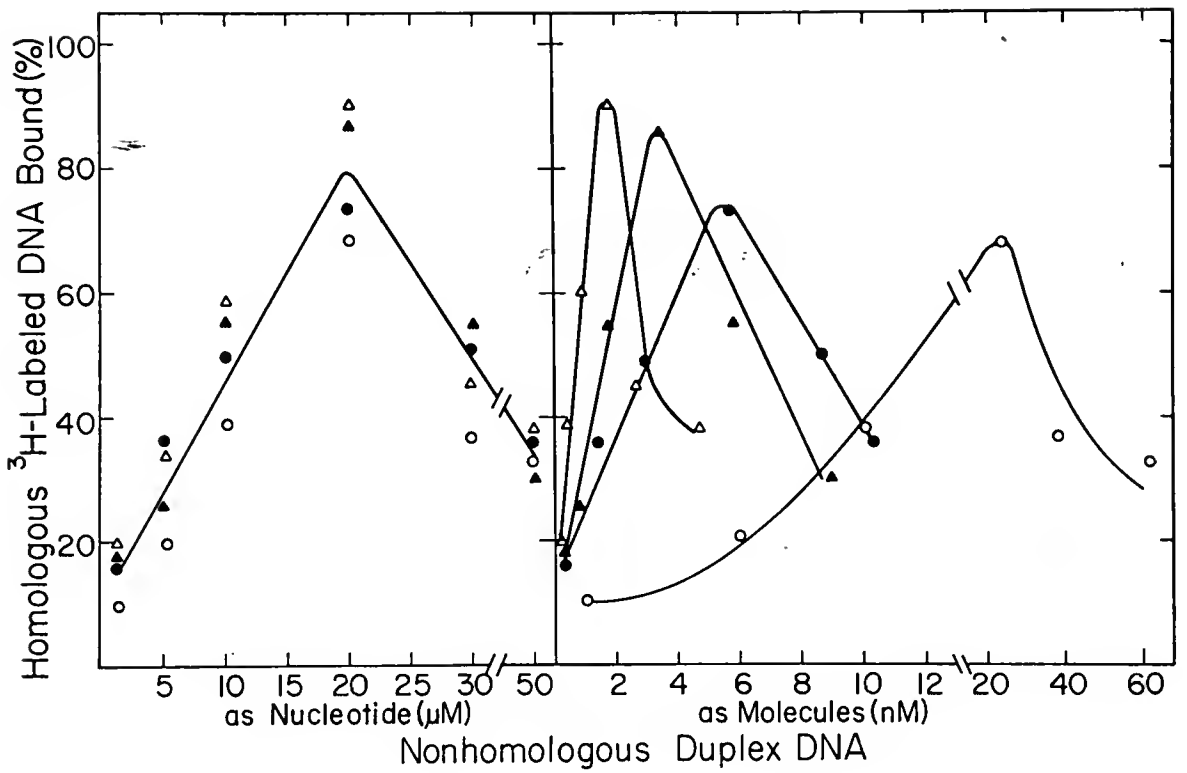
These results suggested to us that maintenance of rec1 activity by duplex DNA occurred through formation of a transient complex in which rec1 protein was protected from inactivation via some molecular interaction with the nonhomologous pair of single and double stranded DNAs. Although such a complex has not been demonstrated directly since the signal depends upon DNA homology, formation of some kind of nonhomologous complex has been observed when a nonhydrolyzable ATP analog is added to pairing reactions. The complex may be akin to the unaligned complex envisioned by Gonda and Radding (1983) which is formed when E. coli recA protein conjoins two DNA molecules but does not place them in homologous register.

As a way to explore the nature of heterologous complex, the stoichiometry between heterologous DNA molecules leading to the highest yield of synaptic complexes between homologous complexes was measured. Yield was optimal when the nucleotide concentration of the single stranded DNA was equivalent to the base pair concentration of the nonhomologous duplex DNA, regardless of length of the duplex molecules (Fig. 6-4).

In another approach, the fate of the circular single stranded DNA through presynapsis and into synaptic complexes was followed under conditions where rec1 protein was protected from inactivation by

Fig. 6-4. Stoichiometry of protector DNA.

Reaction mixtures containing 10 μg/ml rec1 protein, 10 μM single-stranded circular M13 DNA, 1 mM ADP and the indicated concentration of linear duplex DNA fragments of ϕX174 (Sanger et al., 1978) were preincubated at 37°C. After 20 minutes ATP was regenerated, 20 μM ^{3}H-labeled M13 linear duplex DNA was added and incubation continued for an additional 30 minutes. The level of synaptic complexes was measured when the ϕX174 linear duplex protector DNA was 5386 base pair PstI linear duplex DNA (△); 2748 base-pair HpaII fragment (▲); 1697 base pair HpaII fragment (●); 392 base-pair HpaI fragment (○).


Homologous ^{3}H-Labeled DNA Bound (%)
100
80
60
40
20
5
10
15
20
25
30
50
2
4
6
8
10
12
20
40
60
as Nucleotide (μM)
as Molecules (nM)
Nonhomologous Duplex DNA

nonhomologous duplex DNA. Rec1 protein was mixed with ADP and ^{125}I-labeled circular single stranded M13 DNA, but also included nonhomologous ϕX174 linear duplex DNA to prevent presynaptic inhibition. After preincubation, ^{3}H-labeled linear duplex M13 DNA was added. ATP was regenerated from the ADP and challenged with ^{32}P-labeled single stranded M13 DNA. The ^{3}H-labeled synaptic complexes with associated labels formed in the following period was then measured. No ^{32}P-labeled M13 DNA appeared in the final product. Only the ^{125}I-labeled DNA was present (Fig. 6-5A).

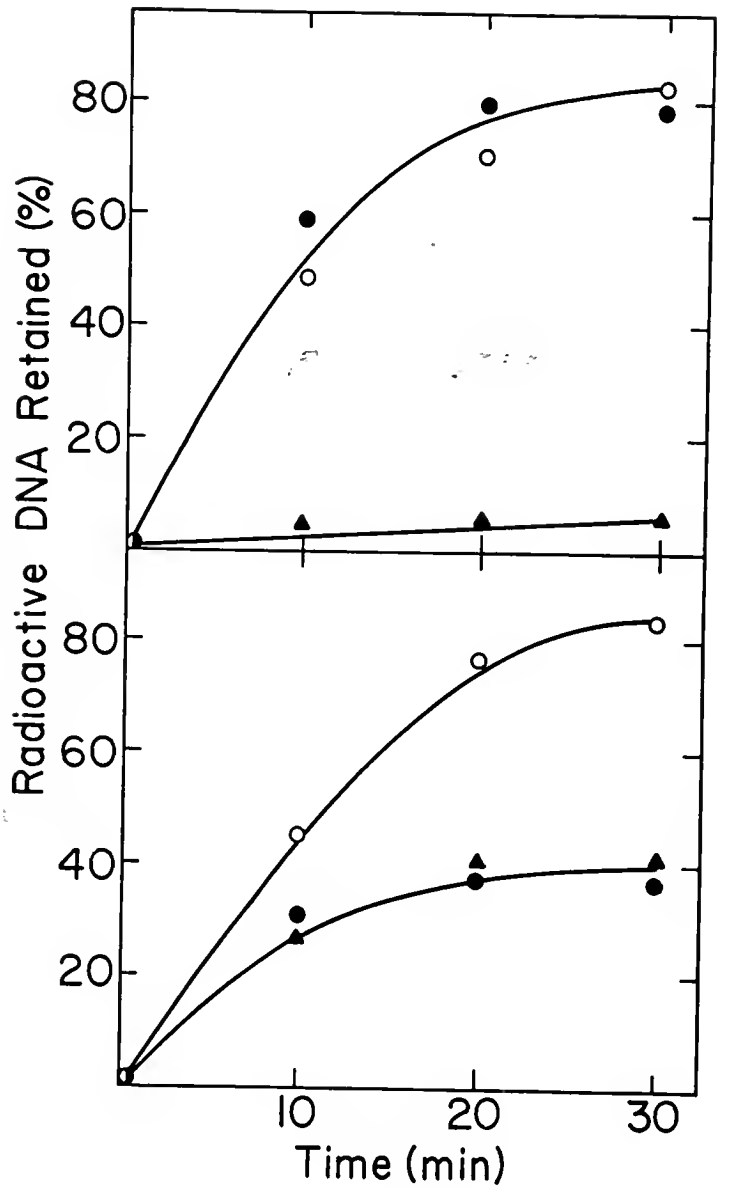
Low Temperature Reverses Presynaptic Inhibition

Presynaptic inhibition is not irreversible. Cold reactivates the presynaptic mixture for synapsis. When rec1 protein inactivated during presynapsis with ADP and single stranded DNA was placed on ice, activity was gradually restored over the course of 20 minutes. At temperatures above 10°C there was little restoration of activity. Unlike duplex DNA, the effect of cold was indeed to reverse the inhibition rather than to prevent it. This slow recovery of activity is suggestive of a conformational change in protein structure induced by low temperature. A plausible interpretation of these results might be that a presynaptic inactive complex formed with rec1 protein, single stranded DNA, and ADP is cold sensitive. It might be expected that reactivation induced by cold follows dissociation of the complex. In a test of this idea the fate of labeled single stranded circular DNA was followed through synapsis and into the synaptic complex, examining if the presynaptic complex could be dissociated by low temperature. Rec1 protein, ADP, and ^{125}I-labeled single stranded M13 DNA were incubated under conditions to inhibit synapsis, the mixture placed on

Fig. 6-5. Fate of presynaptic single-stranded DNA during synapsis.

A. Preincubation reaction mixture containing 7.5 μM ^{125}I-labeled single-stranded circular M13 DNA, 15 μM φX174 linear duplex DNA, 1 mM ADP, and 15 μg/ml rec1 protein was held at 37°C. After 20 minutes ATP was regenerated followed by addition of 7.5 μM ^{32}P-labeled single-stranded circular M13 DNA and 15 μM ^{3}H-labeled M13 linear duplex DNA. Synaptic complexes were measured after 30 minutes.

B. In a second reaction protector duplex DNA was omitted. The mixture was preincubated as above but held on ice for 20 minutes before adding ^{32}P-labeled single-stranded M13 DNA, ^{3}H-labeled linear duplex M13 DNA and regenerating ATP. ^{3}H-labeled linear duplex DNA (○); ^{125}I-labeled DNA (●); ^{32}P-labeled DNA (▲).


Radioactive DNA Retained (%)
80
60
40
20
80
60
40
20
10
20
30
Time (min)

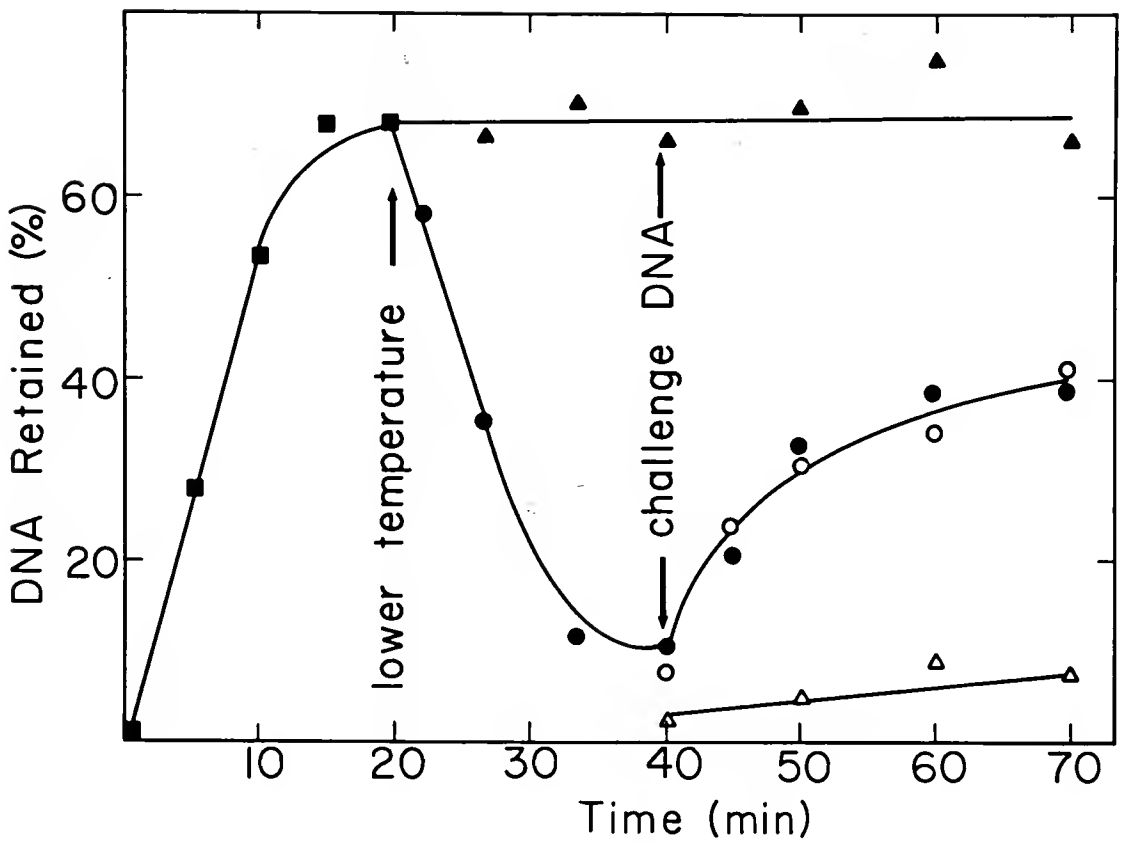
ice for 15 minutes, then [^{3}H]-labeled linear duplex M13 DNA was added, ATP regenerated, and challenged with [^{32}P]-labeled single stranded M13 DNA as before. In this case, stable complexes formed during synapsis contained equal amounts of (Fig. 6-5B) [^{125}I]- and [^{32}P]-labeled single stranded DNAs. A likely explanation is that the complex dissociated at low temperature, whereupon the rec1 protein was reactivated for homologous pairing and could now use either labeled single stranded DNA in the reaction. Direct support for this idea comes from a DNA binding experiment. At 37°C rec1 protein bound tightly to [^{3}H]-labeled single stranded DNA forming a strong complex. When challenged at a later time by addition of [^{32}P]-labeled single stranded DNA, the rec1 protein remained attached to the [^{3}H]-labeled DNA and bound no [^{32}P]-labeled DNA. Lowering the temperature to 0°C dissociated rec1 protein from the complex. When challenged with the second DNA, rec1 protein now bound to both DNAs after the temperature was raised (Fig. 6-6).

Discussion

The experiments outlined in this chapter have provided insight into the events which proceed and apparently control the synapsis of DNA molecules catalyzed by rec1 protein. When the synapsis components of single stranded and double stranded DNA, ATP and rec1 protein were united in various combinations in a reaction prior to synapsis, ternary complexes were formed to the same level as when all the components are incubated together. ADP has been found to inhibit formation and promote dissociation of synaptic complexes. Yet, in a presynaptic reaction mixture it had no negative effect on rec1 protein unless single stranded DNA was also present. Then, synaptic complex formation

Fig. 6-6. Temperature dependent binding of rec1 protein to single-stranded DNA.

A reaction containing 7.5 μM ^{3}H-labeled single-stranded circular M13 DNA, 1 mM ADP, and 15 μg/ml rec1 protein was incubated at 37°C (●). Aliquots were removed at the indicated times and DNA retained on a nitrocellulose filter was determined as in Appendix F. After 20 minutes the reaction was split into two portions. One was placed on ice (circles) and the other maintained at 37°C (triangles). After a further 20 minutes each reaction was challenged with 7.5 μM ^{32}P-labeled single stranded M13 DNA (open symbols) and both mixtures were placed at 37°C.


DNA Retained (%)
60
40
20
10
20
30
40
50
60
70
Time (min)
lower temperature
challenge DNA

was stymied. This presynaptic inhibition can be blocked by addition of nonhomologous duplex DNA to the presynapsis reaction mixture. Alternatively, presynaptic inhibition can be reversed by placing inactivated presynaptic mixtures on ice for 20 minutes.

The reversal of presynaptic inhibition is probably the result of a slow recovery of the protein due to a conformational change. Another possibility is that the single stranded DNA in the presence of recl protein and ADP slowly becomes inactivated and unable to become part of a synaptic complex. This type of cycling is reminiscent of the effect of ice on recA protein-promoted D-loop formation (Shibata et al., 1982b). In this case, recA protein catalyzes the uptake of single strands of DNA by a homologous superhelical duplex, and subsequently promotes their dissociation. The form I DNA becomes inactivated and unable to take part in further D-loop formation. By incubating the reaction mixture on ice-water for 15 minutes, the inactivated DNA is fully reactivated.

The D-loop cycle commences when the recA protein brings the single stranded fragment into the superhelical duplex and begins the search for homologous sequences. The enzyme unwinds the duplex DNA molecule ahead of the pairing fragment. Since recA protein can polymerize upon DNA, long filaments are formed in a polar fashion eventually unwinding the entire duplex. RecA protein is now bound to a duplex molecules lacking superhelical turns, but under torsional strain. The single stranded fragment is displaced. Treatment with either ADP or ice-water causes the dissociation of recA protein from the DNA. The superhelicity of the duplex is recovered and D-loop formation once again possible.

The pathway followed by recA protein in transferring a circular single stranded DNA molecule onto a homologous linear duplex is thought to include several distinct steps. In the first, recA protein slowly polymerizes on the DNA (presynapsis) and this association is stabilized by a single strand binding protein (Cox and Lehman, 1982). The second step involves the interaction with duplex DNA, the search for homology and the breakage and reunion of hydrogen bonds (synapsis). Finally, the heteroduplex is extended, ATP is hydrolyzed and recA protein becomes associated with the duplex. The state of the complex at the end of the reaction is not known, but Cox and Lehman (1982) have reported that the protein is not in a form to interact with added DNA. This is contrary to experiments presented by Shibata et al. (1982) in which recA protein was capable of carrying out D-loop formation after becoming dissociated from duplex DNA. Perhaps, different mechanisms of DNA pairing take place with different DNA substrates.

An intriguing question about the initiation of pairing reactions, like the formation of a D-loop or the strand transfer reaction concerns the manner in which the enzyme searches for DNA sequence homology and moves through regions of nonhomologous DNA. Recently, Gonda and Radding (1983) studied this by using circular single stranded DNA and linear duplex molecules containing varying amounts of nonhomologous sequences. They observed that heterologous DNA sequences accelerate the homologous pairing event. This, they concluded, was evidence for a processive search for homology carried out by the recA protein.

Two mechanisms for processive movement on DNA are possible. One encompasses a group of random collisions within the ternary complex, while another envisions an active sliding mechanism in which the two

DNA molecules move by each other. Once homologous sequences are in register, pairing can begin. Other proteins such as the lac repressor (Winter et al., 1981) and EcoRI endonuclease (Jack et al., 1982) have provided precedents for such processive movement on DNA.

Our understanding of the method used by recl protein in the pairing reactions it catalyzes is currently only speculative. However, a pattern and sequence of reactions is becoming more clear as the data becomes available. The pairing reaction can be broken down into three phases of which the first, presynapsis, is the most susceptible to inhibitory factors such as ADP. During this phase, recl protein may bind to single stranded DNA without the energy requirement of ATP. However, once attached the enzyme becomes sensitive to inactivation by ADP. The subsequent enzymatic movement to the duplex DNA molecule brings together the ternary synaptic complex. Recl protein holds the DNA molecules together at nonhomologous sites until the search for homology begins, promoted by ATP. This ternary complex prevents ADP from inactivating the recl protein.

The fact that ADP only inactivates recl protein attached to single stranded DNA suggests two reaction properties. First, the recl protein may change its conformation upon interaction with single stranded DNA making it more liable to ADP inactivation. Second, the formation of an active recl proten single strand DNA complex is the rate limiting step in strand transfer reactions. These observations also suggest a regulatory role for ADP in controlling the initiation of recombination. All recombination intermediates created in vitro by the enzymatic activity of recl protein require single stranded DNA. The regulation of active presynaptic complexes may therefore control overall

recombination events. Because the ADP inactivation does not involve dissociation from DNA, the enzyme may be allowed to function in other ways such as preventing single strand regions of DNA from becoming digested by endogenous nucleases. The complex but fascinating nature of the recl protein is slowly becoming unveiled but its regulation remains a mystery. The molecular interactions which modulate its regulation remain unexplained.

CHAPTER SEVEN
INDUCTION OF REC1 PROTEIN BY U.V. IRRADIATION OR HEAT SHOCK

When a variety of organisms are exposed to adverse stimuli, such as a sudden shift in temperature or irradiation by ultraviolet light (U.V.), they often respond by inducing or accelerating protein synthesis. These proteins are usually involved in the regulation of cell division, although much speculation still exists about them. Heat shock proteins have been isolated from a number of different organisms (Lindquist, 1980b; Neidhardt and VanBogelelen, 1981; Ballinger and Pardue, 1983). Similar responses can be induced by other pertubatory conditions. A complex series of metabolic reactions is triggered in response to DNA damaging U.V. irradiation. This type of cellular pertubation leads to single strand breaks in the DNA which have been found to indirectly stimulate recombination. In proficient cells the level of recA protein is increased 20- to 50-fold after U.V. irradiation (Salles and Paoletti, 1983). The recA protein serves to cleave the lexA repressor protein, a negative modulator of recA protein (Gudas and Pardee, 1976). It also catalyzes DNA repair via a recombination repair pathway. This SOS response, which include an array of other proteins (uvr ABC) (see Sancar and Rupp, 1982), has been well documented in prokaryotes. However, the response of eukaryotic cells to various cellular stresses has not been well characterized. Initial studies have suggested that rearrangement of mammalian chromatin occurs as a response to excision repair (Zolan et al., 1982). In addition,

several enzymes, analogous to the uvr A, B, C, gene products have been tentatively identified (Dresler and Lieberman, 1983).

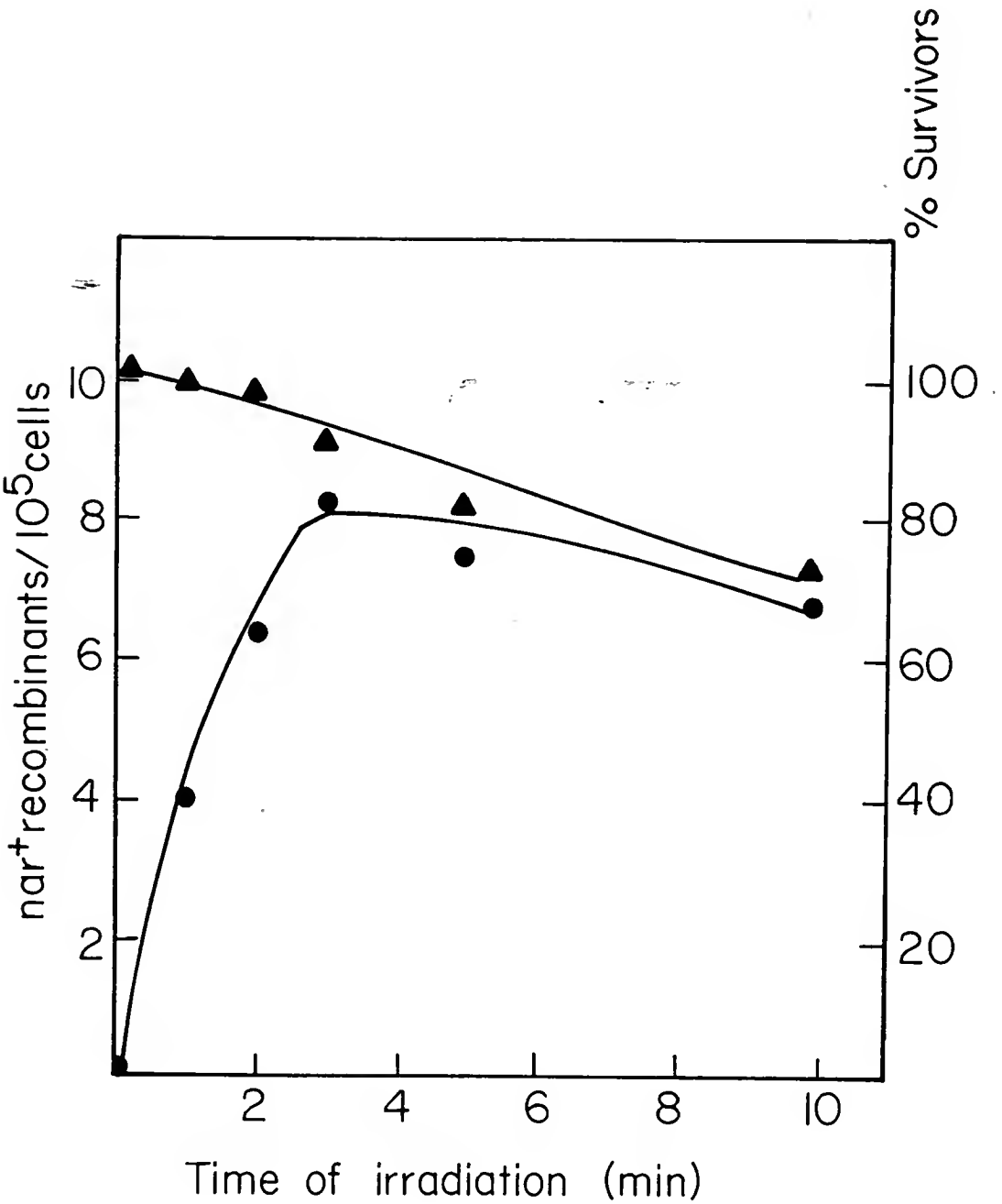
Introduction of strand breaks in fungi is known to strongly enhance the rate of mitotic recombination (Game et al., 1979). Induction of mitotic recombination after irradiation can be prevented by blocking protein synthesis (Holliday, 1975).

In Ustilago maydis, the rec1 mutant, which is defective in repair of damage by U.V. light, is altered in recombination pathways as well (Holliday et al., 1976). Evidence for inducible DNA repair process in Ustilago maydis has been presented (Lee and Yarrington, 1982). Taken together these observations can be interpreted to mean that the rec1 gene product may regulate both processes as part of an SOS response. The rec1 protein has been shown to carry out a variety of DNA strand transfer reactions, yet little data has been attained which would implicate it in cellular regulation pathways. To more closely examine Ustilago maydis' response to cellular stresses, in the hope of inducing higher production of rec1 protein, the cells were irradiated with U.V. light or exposed for a short period of time to higher temperatures.

Irradiation of Ustilago Cells

The kinetics of induction of rec1 protein using a rec1$^+$ diploid containing a heteroallelic nar locus were followed. This strain allows for correlations between mitotic recombination and rec1 protein activity to be made. Intragenic recombination will cause the strain to revert to a nar$^+$ phenotype which can easily be scored for by the utilization of nitrate as the role source of nitrogen. This event is due to gene conversion, not reciprocal recombination, and is greatly enhanced by U.V. irradiation (Holliday, 1966). For the optimal dose of

Fig. 7-1. Induction of recombinants by ultraviolet light.
Cells resuspended in 8 liters of water at 2.3 x 10^8 cells/ml were irradiated with a submersible ultraviolet lamp. Aliquots were removed at the indicated times and diluted samples (0.1 ml) were spread on nitrate minimal medium to determine recombination or on YEPS to determine surivival. Recombinants (●); viability (▲).


% Survivors
nar+recombinants/10^5cells
10
8
6
4
2
100
80
60
40
20
2
4
6
8
10
Time of irradiation (min)

U.V. light to use in the induction experiments, the level of radiation which yielded a high level of gene conversion with maximal cell survival was determined. As illustrated in Figure 7-1, 3 minutes was found to be optimal, yielding a high level of recombinants with little cell killing.

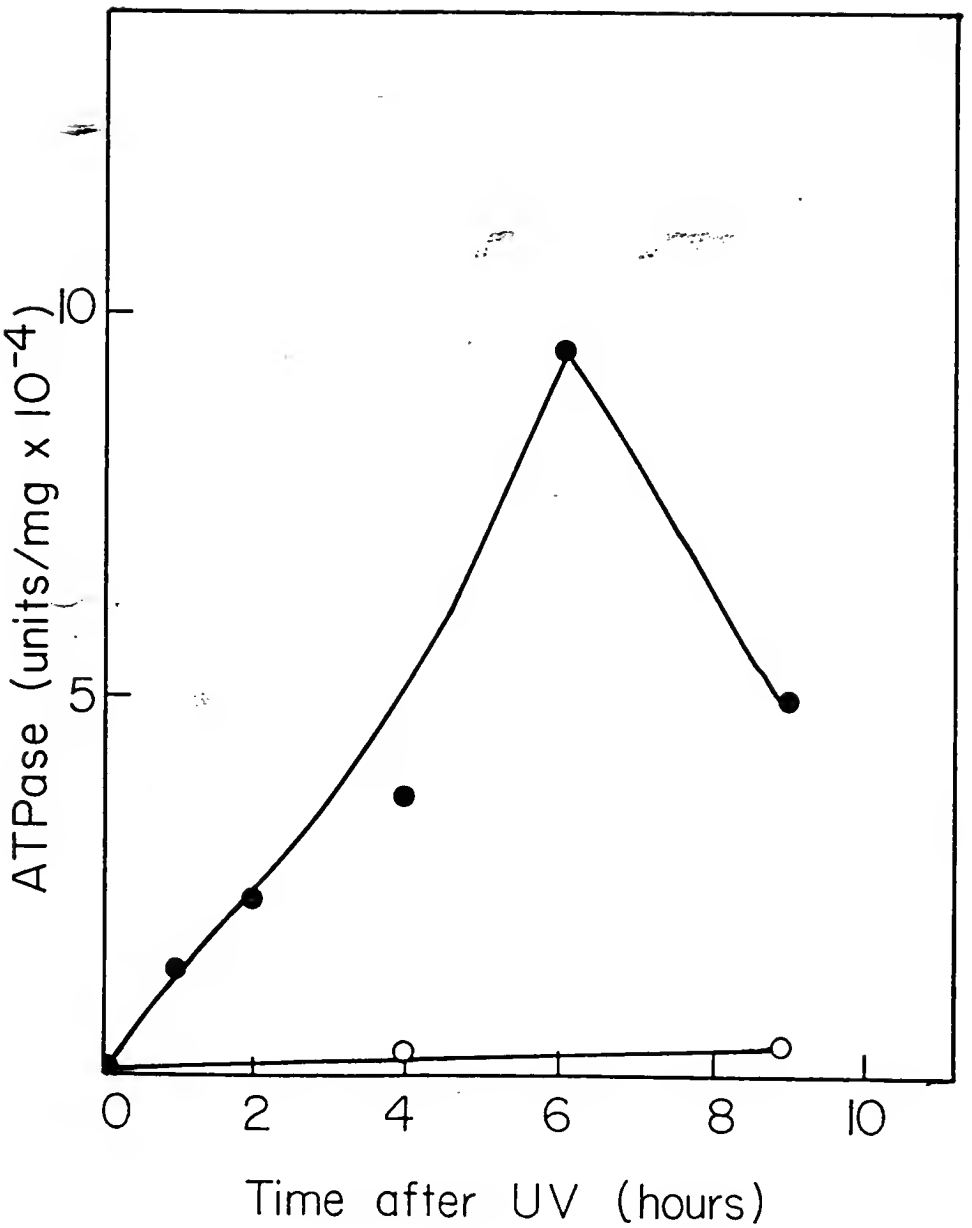
A comparable batch of cells was irradiated and the level of rec1 protein induced monitored. Assays for rec1 protein are not reliable if the protein fraction is impure. Therefore, rec1 protein was purified through the phosphocellulose step (see Chapter 2). This purification was carried out quickly for fear of a concurrent induction of repressor or repressor-like activities which would control rec1 protein synthesis. Figure 7-2 depicts the results. A substantial increase in the level of DNA-dependent ATPase activity in cells irradiated with U.V. light was observed. The level of activity rose progressively with time, peaking at 6 hours, then falling off. The maximal level achieved was some 20-fold higher than uninduced cells.

Heat Shock of Ustilago Cells

The purification of recA protein has been achieved by a variety of laboratories (Cunningham et al., 1982; Cox et al., 1982). Since the same purificaiton scheme is used when isolating high amounts induced recA protein, this procedure was employed to purify a similar activity from heat-shocked Ustilago cells. This was done in the hope of isolating high levels of rec1 protein, induced by heat shock. Previous attempts to isolate high levels of rec1 protein from heat shocked cells using the normal purification scheme were unsuccessful (data not shown).

Fig. 7-2. Induction of ATPase activity in cells irradiation with UV light.

Cells were grown and irradiated as described in Appendix A. At time zero cells were irradiated for 3 minutes. Cultures were continued for the indicated periods of time before harvesting for determination of recl activity. Irradiated (•); unirradiated (o).


ATPase (units/mg x 10^{-4})
10
5
0
2
4
6
8
10
Time after UV (hours)

High Speed Pellet (resus.)
(supe.)

Polymin-P Precipitation (0.04%)
(pellet)

50% AmSO4 Precipitation
(pellet)

DEAE Chromatography
(0-1 M NaCl Gradient)
(Elution at 0.25 M)

Single Stranded DNA Cellulose Chromatography
(Flow through)

Phosphocellulose Chromatography
(0-1 M KCl Gradient)
(Elution at 0.25 M)

Dialysis
(Removed of Salt)

Fig. 7-3. Purification of 110 K Protein

The purification protocol outlined in Figure 7-3 was used after normal crushing and resuspension of Ustilago cells in 1 molar NaCl. The cells were grown for 30 hours at 32°C. The temperature was then raised ro 42°C for 30 minutes and once again lowered. Growth at 32°C continued for 30 minutes and the cells harvested. Reannealing and DNA-dependent ATPase activity were monitored as described before. Both activities co-chromatographed on phosphocellulose eluting at approximately 0.35 molar NaCl. This corresponded to the elution profile from phosphocellulose chromatography in normal rec1 protein preparations (Fig. 7-4). This fraction was dialyzed to remove NaCl and strand transfer reactions carried out. In addition, polyacrylamide gel electrophoresis in 0.01% SDS was run to visualize the proteins present. Two major bands were apparent after staining with silver-nitrate and visualization with sodium bicarbonate. The molecular weights were approximated at 110 kilodalton and an undetermined size in the high molecular weight range. There was no protein band with a molecular weight of 70,000 daltons, characteristic of rec1 protein. In concert with this result is the finding that the DNA strand transfer reaction had a preference for overhanging 5' ends on the duplex linear molecules.

[^{3}H]-labeled fd RFI DNA was digested with either HpaI or Sau96I restriction enzymes. HpaI endonuclease leaves blunt ends on the linear duplex molecule after cleavage, while Sau96I leaves 5' overhangs. When fd single stranded circles were incubated with the 110 k dalton protein and either type of linear duplex described above, only the 5' overhanging duplex was retained in washes of high salt on nitrocellulose filters (Fig. 7-5). No retention of linearized DNA was observed

Fig. 7-4. Phosphocellulose chromatography: Reannealing Activity and ATPase.

A phosphocellulose column (1 x 12 cm) was loaded, washed and eluted as described in Appendix D. The flow rate was maintained at 30 ml/hour, and fractions of 2 ml were collected. Activity determined as described in Appendix E.

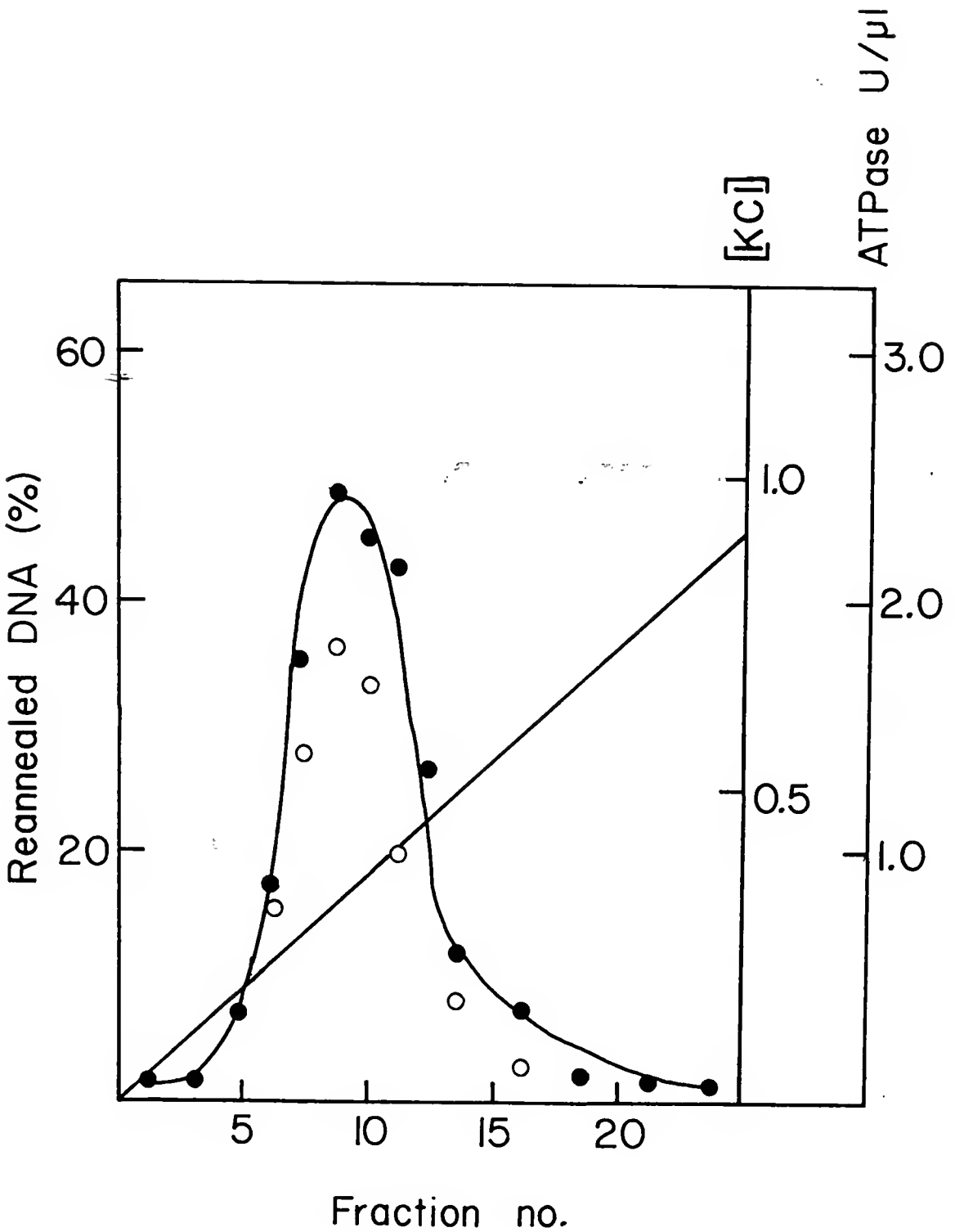
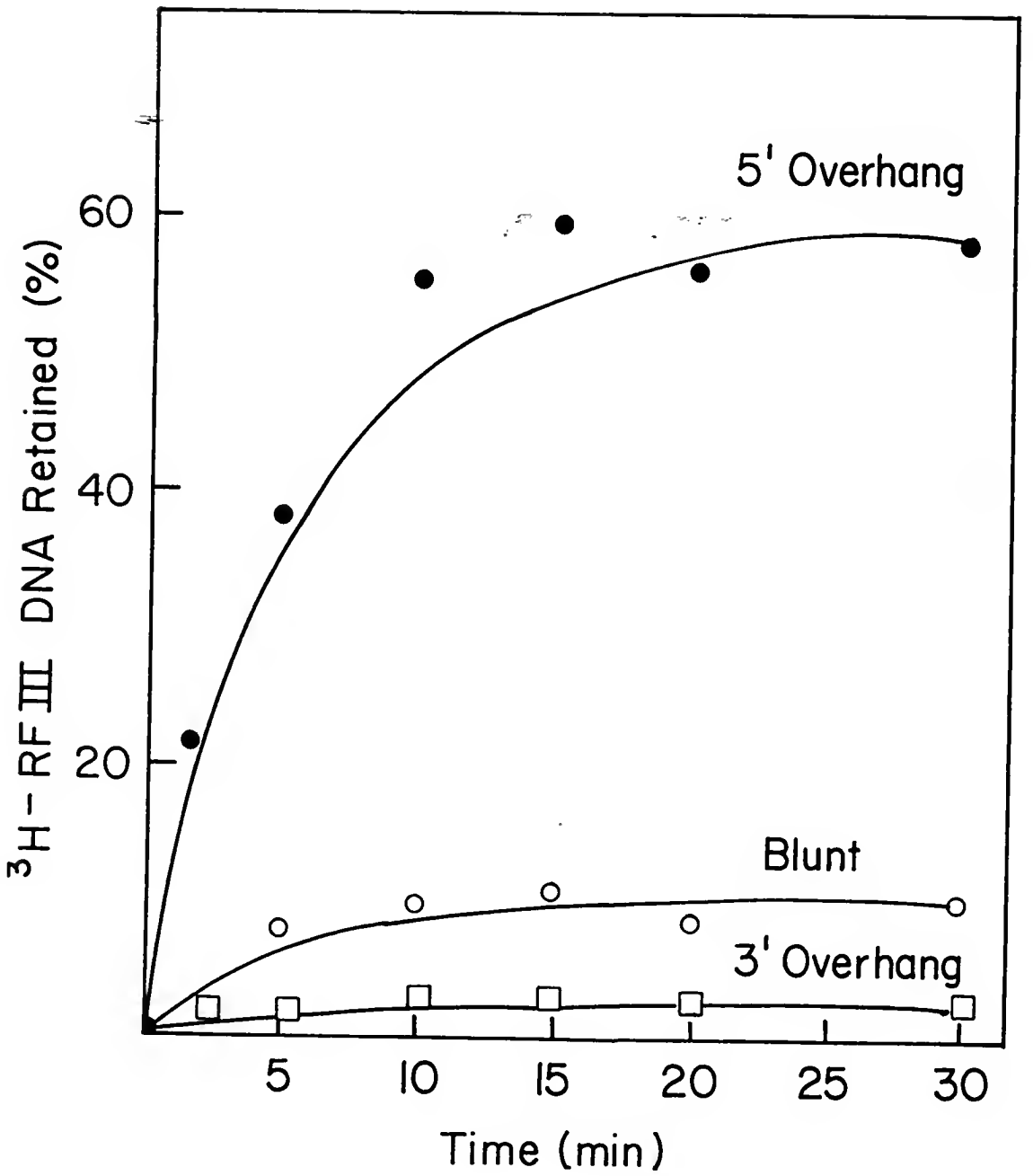

Reannealed DNA (%)
60
40
20
5
10
15
20
Fraction no.
[KCl]
1.0
0.5
ATPase U/µl
3.0
2.0
1.0

Fig. 7-5. The effect of end structure in strand exchange reactions. Reactions mixtures (350 µl) containing 15 M of either Sau96I digested (5' overhanding ends). HpaI digested (blunt ends), or EcoRII digested (large fragment) (3' overhanging ends) ^{3}H-labeled fd linear duplex DNA, 7.5 µM fd single strand circular DNA and 15 µg/ml recl protein were incubated at 37°C. At indicated 50 µl aliquots were removed and processed as described in the D-loop assay (Appendix F).


5' Overhang
Blunt
3' Overhang
^{3}H-RF III DNA Retained (%)
60
40
20
5
10
15
20
25
30
Time (min)

when the molecule was blunt-ended or possessed 3' overhanging ends. This is not the result observed when recl protein is used to catalyze these strand transfer reactions, although there does seem to be a slight stimulation when duplex linear molecules with 5' overhangs are used.

Discussion

The results described in this chapter outline the attempts made at producing increased levels of recl protein. Normal purification procedures yield approximately 100 µg of pure protein, isolated from 1500 grams of packed cells. Neither U.V. light irradiation nor heat shock stress induced the production of increased levels of recl protein. However, U.V. irradiation did significantly increase the ATPase activity of recl protein. This level reached a maximum 6 hours post-irradiation. Holliday (1971) reported that recombination is complete 2 to 4 hours after irradiation. This time discrepancy is intriguing. The fact that recl protein was induced to increased levels of activities from 1 to 6 hours post-irradiation may implicate it in the repair process. It may also participate in another processing event occurring somewhat later in the irradiated cell.

The results of these experiments, in accord with Lee and Yarrington (1982), suggest that no novel protein bands were detected after irradiation. These investigators, however, observed the loss of several protein bands. Taken together, one may postulate that one of these proteins may be a repressor of recl protein activity. Once removed, by irradiation, recl protein activity increases. On the other hand, this crude measurement of induced protein synthesis may not avail itself for such conclusions. In E. coli it is known that at

least 6 genes are expressed in response to DNA damage (Walker, 1981), but only one gene product is observed by SDS-polyacrylamide gel electrophoresis. The complex system of repair in prokayrotes may be only a shadow of the type of mechanisms ongoing in eukaryotes. Still, the existence of a repressor protein could explain the relatively low levels of recl protein obtained in homogeneous state.

Putting Ustilago cells under a stressful heat condition did not increase the yield of recl protein. However, using a purification scheme similar to that used in recA protein purification, an interesting enzyme was purified to near homogeneity. The reannealing, ATPase and column elution profiles are all quite similar to those of recl protein. An important difference is seen by the preference for 5' overhanging ends in the strand transfer reactions and the molecular weight. This corresponds to the polar movement of heteroduplex growth promoted by recl protein (Kmiec and Holloman, 1982). However intriguing this observation may be, no direct correlation to the recl protein can be made. The fact that Ustilago possesses two proteins capable of carrying out similar recombination activities in response to two different stressful environments suggests that the organism has developed a complex system of response, but the nature of that response remains to be determined.

CHAPTER EIGHT
THE INTERACTION OF REC1 PROTEIN WITH LEFT-HANDED Z-DNA

Z-DNA is a left-handed form of the DNA helix favored thermodynamically in sequences of alternating purine and pyrimidine residues. Increased negative supercoiling of a circular double helix favors the right (B)- to left-handed (Z) structural transition (Singleton et al., 1982; Peck et al., 1982). A wide range of DNA molecules have been created which theoretically contain stretches of Z-DNA. Peck et al., (1982) constructed plasmids which contain regions of alternating dC-dG residues and detected the B to Z transition by alterations in sedimentation deficients and gel electrophoresis mobilities. These investigators showed that a plasmid containing a 32 base pair insert forms Z-DNA segments at a lower superhelical density than one containing only a 14 base pair insert. Studies with Z-DNA specific antibodies (Nordheim et al., 1983) confirmed the presence of left-handed helices within the inserted region.

Another source of left-handed helices within DNA is form V DNA, an unlinked covalently closed circular double stranded molecule (Stettler et al., 1979). Because of the torsional strain within this duplex, right-handed helices must be compensated by supercoiling and left-handed helices. Until recently this type of molecule was the only known form of DNA having a natural sequence that displayed stretches of Z-DNA.

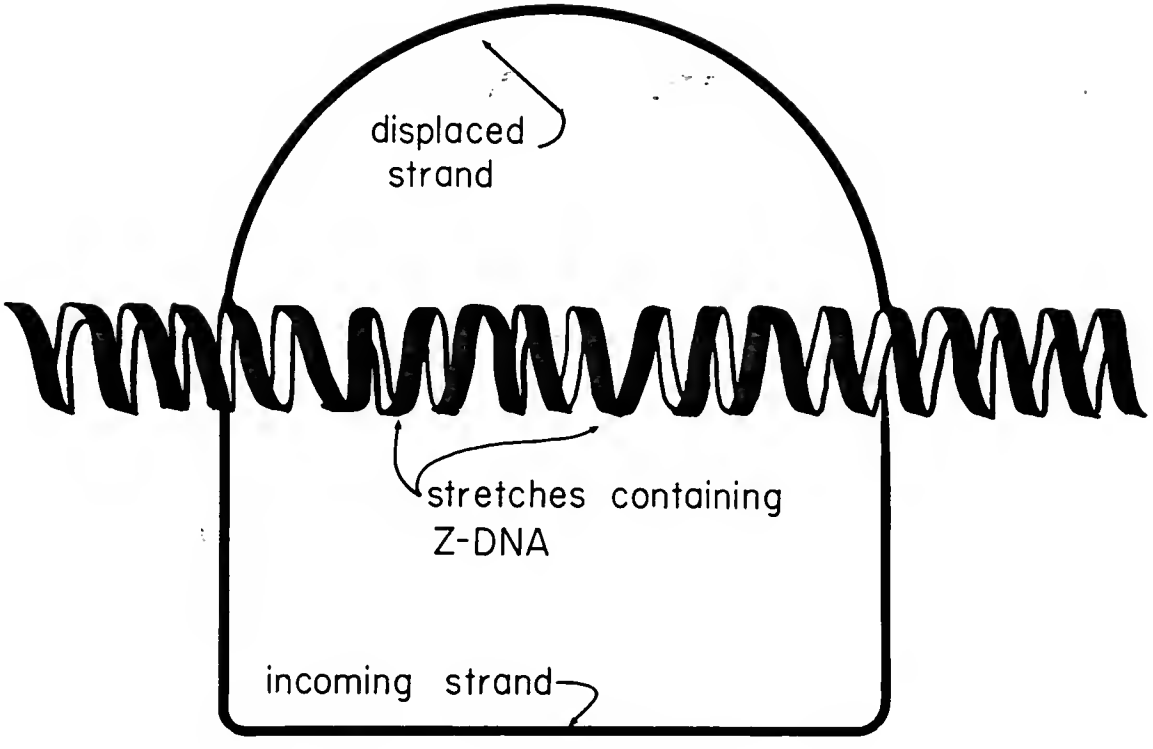
A definitive biological role for Z-DNA has not been found. Several reports have indicated that regions in Drosophila polytene chromosomes react with Z-DNA antibodies (Nordheim et al., 1982; Viegas-Pequignot et al., 1983). Potential Z-forming regions of d(Tp6)n have been found widespread in eukaryotic DNA sequences. An intron of a human cardiac muscle actin gene (Hamada and Kakunaga, 1982) and regions of the yeast genome (S. cerevisiae) (Walmsely et al., 1983) contain long stretches of putative Z-DNA sequences. Recently, telomeric regions of chromosomes have been found to hybridize with putative Z-DNA probes. This particular result suggests a regulatory role for left-handed DNA as telomeres are thought to participate in the initiation of replication. The simian virus 40 (SV40) enhancer region, thought to be a site of transcriptional control, has been shown to form Z-DNA on negative supercoiling (Nordheim and Rich, 1983).

The recl protein has been shown to promote homologous pairing of two DNA molecules in the absence of a free end (see Chapter 5). The structure formed contains regions of left-handed DNA, recognized by Z-DNA antibodies. This paranemic joint (Fig. 8-1) is unstable and sensitive to dissociation by ADP. The junctions between the B- and Z-DNA segments have been found to be conformationally flexible (Singleton et al., 1983) and may intrinsically contribute to dissociation. To further characterize the interaction between recl protein and Z-DNA, experiments were designed to examine the binding of left-handed DNA by recl protein and the parameters surrounding such interactions.

Z-DNA was made as previously described (Kmiec and Holloman, 1984) and labeled at the 5' end with γ-^{32}P ATP using bacterial alkaline phosphatase and T-4 kinase. The specific activity of this DNA was 1 x

Fig. 8-1. The paranemic joint.

Diagramatic representation of a paranemic jointed formed when a single stranded circular DNA molecule pairs with a linear duplex at homologous sites located within the duplex, not at the ends. Putative locations of Z-DNA are depicted.


displaced
strand
stretches containing
Z-DNA
incoming strand

10^7 cpm/μmole and contained alternating dG·dC residues. The presence of the left-handed helical form was confirmed by a negative circular dichroism measurement at 290 nm. The DNA in the Z-form was assayed for reactivity with Z-DNA specific antibodies. Left-handed DNA labeled at the 5' end with [^{32}P]ATP but not B-DNA was bound by the antibodies.

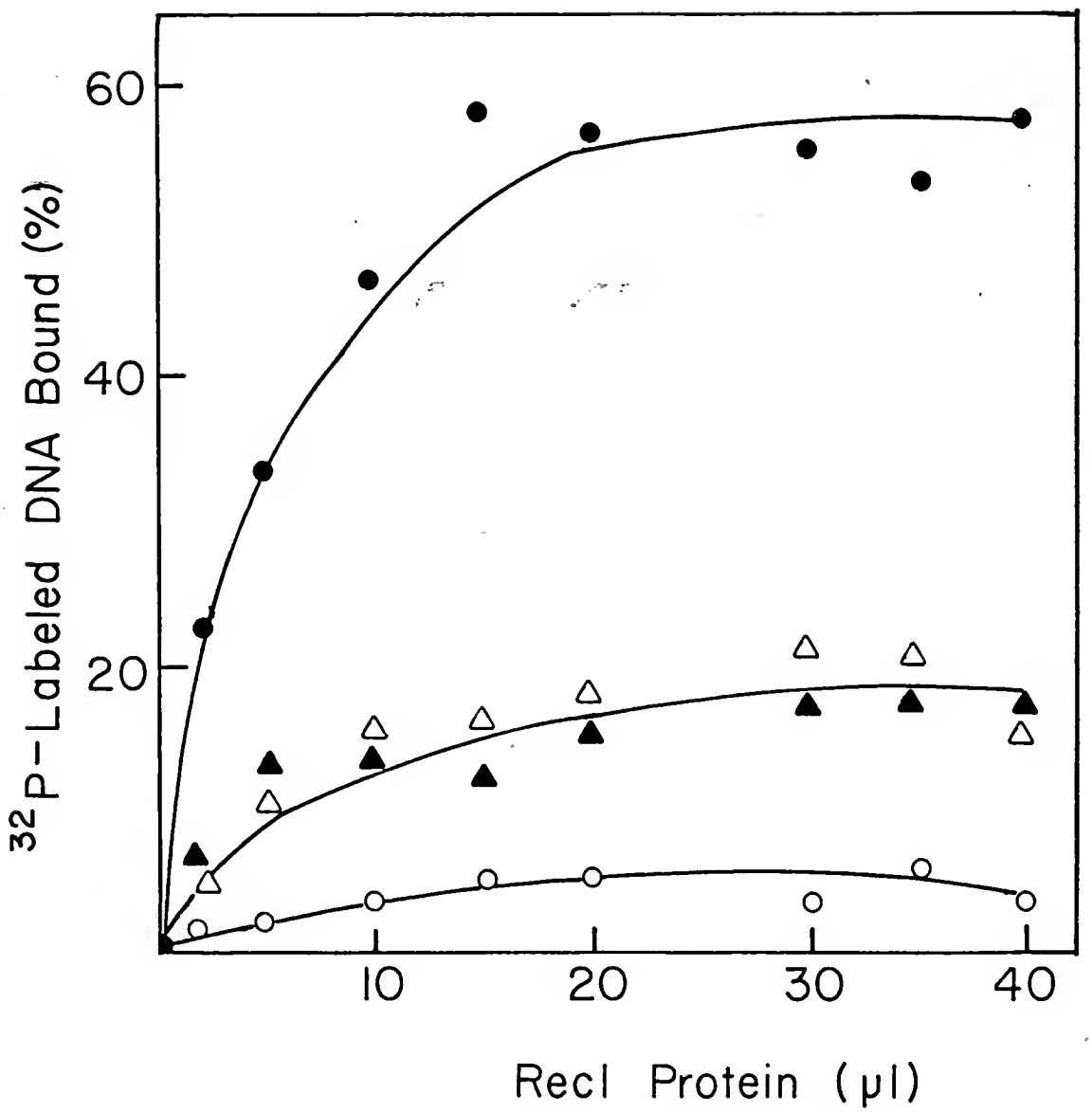
The binding of Z-DNA by rec1 protein was determined using the standard nitrocellulose filter binding assay for protein bound DNA. Reaction mixtures contained rec1 protein, ATP and ^{32}P-labeled poly[dG·dC] in either the B- or Z-form. As depicted in Figure 8-2, the polymer in the Z-form was bound rapidly and to high levels by increasing concentration of enzyme. The B-DNA form was bound at very low levels. The importance of ATP in the binding of Z-DNA is also illustrated in Figure 8-2. ADP did not serve as a substitute for ATP in binding reactions. When increasing concentrations of ADP were added to reaction mixtures containing rec1 protein and 1 mM ATP, binding of Z-DNA was inhibited but only by 50% at concentrations of 1 mM or greater (data not shown). These results indicate that rec1 protein binds tightly to Z-DNA and cannot be completely dissociated at high concentrations of ADP.

Single Stranded DNA Outcompetes Z-DNA for Rec1 Protein

The binding of single stranded DNA is thought to be the initial step in the homologous alignment of DNA molecules. Since the binding of duplex DNA in the Z-form closely resembles that of single strand DNA, an experiment was designed to determine which substrate was preferred by the enzyme. Rec1 protein was reacted with ^{32}P-labeled Z-DNA in the presence of ATP and the ATP regeneration system. After

Fig. 8-2. Binding of Z-DNA requires ATP.

Reaction mixtures (50 µl each) containing 1 µmole of ^{32}P-labeled poly(dG·dC) (2×10^7 cpm/µmole) in the brominated Z, or unbrominated B, form, with either 1 mM ATP or ADP or neither and indicated amounts of rec1 protein were incubated at 37°C. After 45 minutes reactions were stopped by addition of EDTA to 25 mM. The amount of labeled DNA bound by protein was determined as described in Appendix E. (●), Z-DNA plus ATP; (○), B-DNA plus ATP: (△), Z-DNA plus ADP: (▲), Z-DNA alone.

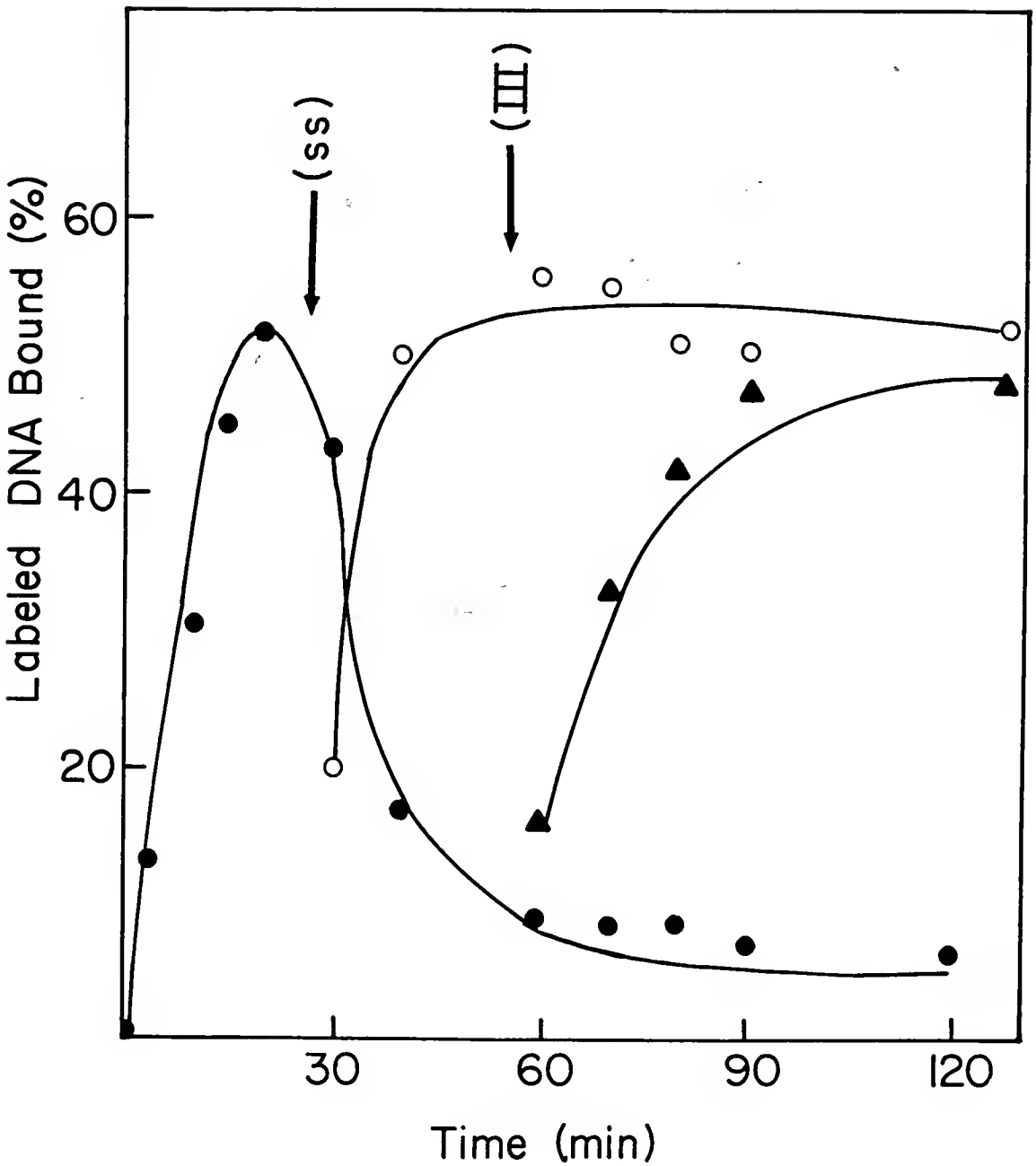

60
40
20
^{32}P-Labeled DNA Bound (%)
10
20
30
40
RecI Protein (µl)

rec1 protein was bound to Z-DNA ^{125}I-labeled single stranded DNA was added. Figure 8-3 illustrates that the retention of Z-DNA decreased while single stranded DNA was bound to a high level. Several possible explanations could be put forth to describe these results. Firstly, the single stranded DNA was bound by unbound rec1 protein, free in solution. The decrease in the level of Z-DNA bound to the filter could simply be due to dissociation. This is unlikely since earlier experiments indicated that the binding of rec1 protein to Z-DNA was firm and undissociable in the normal reaction mixture. A more attractive hypothesis is that single stranded DNA was bound by rec1 protein initially attached to Z-DNA, and therefore can successfully compete for the protein from duplex DNA in the Z-form. This suggests that the binding affinity for single stranded DNA is greater than that of duplex DNA. Since both labels were not simultaneously retained by the filter, a ternary complex is probably not made. This is consistent with previous observations that complex formation requires sequence homology (see Chapter 5).

This experiment was extended one step further. To examine the single stranded DNA-rec1 protein complex for pairing activity, ^{3}H-labeled homologous duplex linear DNA was added after the circles were bound to their highest level. The results of this experiment (Fig. 8-3) indicate that synaptic complexes were formed and that the single stranded DNA-rec1 protein complexes are in an active conformation, capable of participating in homologous pairing. When ^{3}H-labeled nonhomologous duplex linear DNA was added, no ternary complexes were formed.

Fig. 8-3. Rec1 protein has a higher binding affinity for a single stranded DNA versus Z-DNA.

A reaction mixture of 600 µl containing 20 µM of ^{32}P-labeled Z poly(dG·dC) in the brominated form (Z-DNA), 1 mM ATP and the ATP regenerating system, and 15 µg/ml rec1 protein was incubated at 37°C. At 28 minutes of reaction time, 20 µM ^{125}I-labeled single-stranded M13 DNA was added and reaction continued at 37°C. At 58 minutes of reaction time 40 µM of ^{3}H-labeled M13 linear duplex DNA was added and reaction continued at 37°C. At indicated times, 50 µl aliquots were removed, reactions terminated by addition of EDTA to 25 mM, and labeled DNA bound by protein determined as described in Appendix E. (●), Z-DNA; (○), single stranded DNA; (▲), duplex linear DNA.


(ss)
(III)
Labeled DNA Bound (%)
60
40
20
30
60
90
120
Time (min)

Binding of Z-DNA is Stimulated by B-DNA

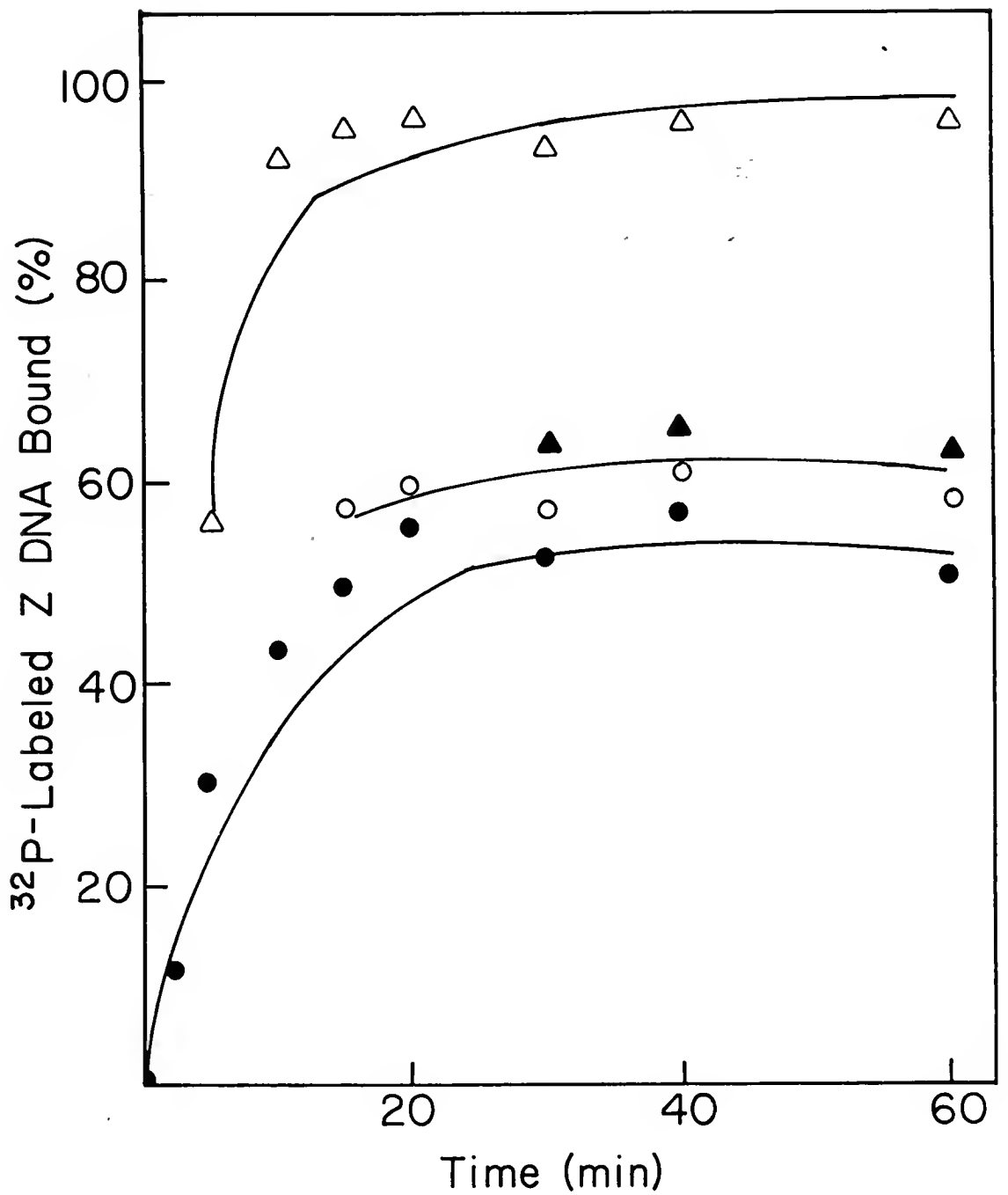
The parameters surrounding the binding of left-handed DNA were examined further. Reaction mixtures containing rec1 protein, Z-DNA, ATP and the ATP regenerating system were incubated at 37°C. At various times, an equal concentration of the polymer dG·dC in the B-form was added. As shown in Figure 8-4, when right-handed DNA is added at 3.5 minutes of reaction time, the binding of Z-DNA is increased several fold. This stimulation did not occur when the B-DNA was added 10 or 15 minutes after the reaction began. These results suggest that B-DNA can stimulate the binding of Z-DNA in much the same manner in which single stranded DNA stimulates the binding of B-DNA. Since this stimulatory effect occurs only within a narrow window of reaction time, there may be a transitory association with B-DNA causing conformational change in the enzyme. This structural alteration may increase the affinity of rec1 protein for Z-DNA.

Poisoning of Paranemic Joint Molecule Formation is Blocked by Z-DNA

It was previously shown that rec1 protein can recognize homology and promote pairing of a circular single strand with an internally homologous region of a linear duplex flanked by sections of nonhomology. The paranemic joint, as described above, contains regions of Z-DNA which are produced when the circle pairs but does not interwind with its complementary strand in the duplex. Furthermore, it was shown that the poisoning of rec1 protein by single stranded DNA and ADP could be blocked by addition of nonhomologous duplex during the presynapsis phase. An experiment was now designed to see if left- handed Z-DNA could block presynaptic inhibition when the heteroduplex joint formed at synapsis was paranemic. In other words, could Z-DNA rescue synapsis

Fig. 8-4. DNA in the B-form stimulates the binding of DNA in the Z-form by rec1 protein.

A reaction mixture of 450 µl containing 20 µM ^{32}P-labeled poly(dG·dC) in the brominated form, Z-DNA, 1 mM ATP and ATP regenerating system, and 15 µg/ml rec1 protein was incubated at 37°C. At 3.5, 10, or 20 minutes of reaction time respectively, 20 µM poly(dG·dC) in the unbrominated form, B-DNA, was added and reaction continued at 37°C. At indicated times, 50 µl aliguots were removed and reaction terminated by addition of EDTA to 25 mM, and labeled DNA bound by protein determined as described in Appendix E. (●), Z-DNA; (△), Z-DNA after addition of B-DNA at 3.5 minutes, (○), 10 minutes, and (▲), 20 minutes, respectively.


100
80
60
40
20
^{32}P-Labeled Z DNA Bound (%)
20
40
60
Time (min)

when there was no topological constraint in the homologous pairing at synapsis

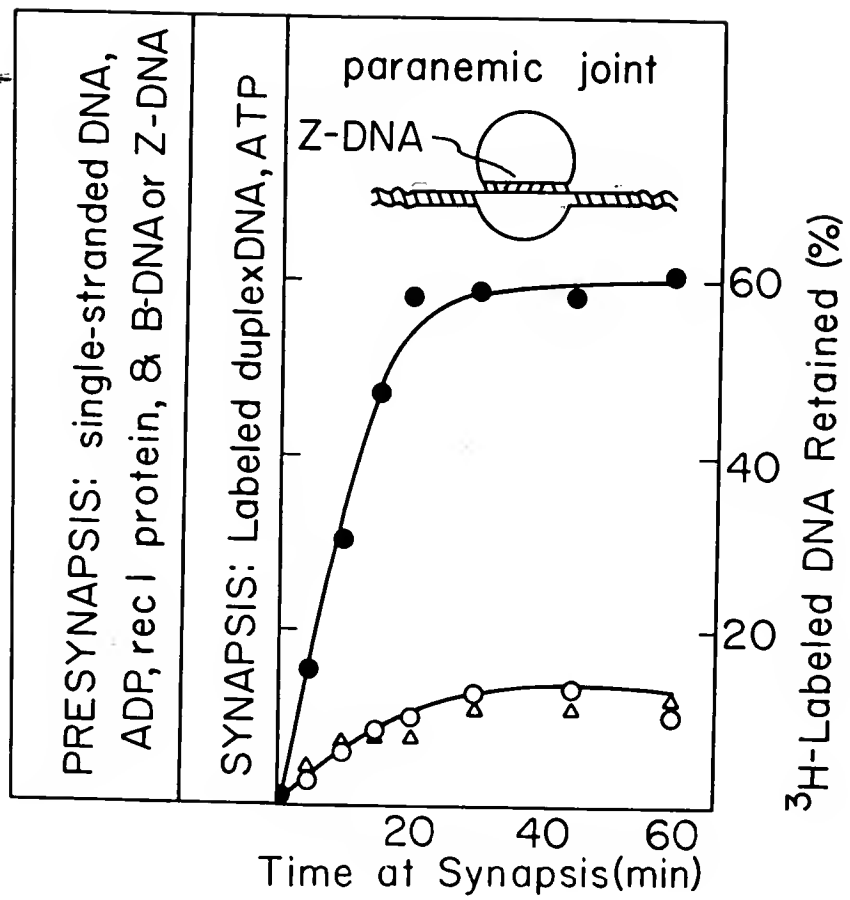
The presynapsis reaction mixture contained poly dG·dC in the B- or Z-form, or nonhomologous ϕX DNA, rec1 protein, single stranded circular DNA and ADP. At synapsis, ^{3}H-labeled M13 Gori1 DNA linearized by BamHI or XhoI digestion and the ATP regenerating system was added, the reaction proceeded and synaptic complex formation measured. The results are shown in Figure 8-5. Synaptic complexes formed between single stranded circular M13 DNA and the ^{3}H-labeled linear duplex M13 Gori1 DNA homologous at internal sites only, when the preincubation contained Z-DNA as the protector duplex DNA. The B-form of the polymer was not effective, nor was linear duplex ϕX DNA. Thus, when DNA topology limits formation of nascent heteroduplex to the noninterwound paranemic joint which contains Z-DNA, the only Z-DNA can protect rec1 protein from presynaptic inactivation.

Discussion

The experiments described in this chapter show that rec1 protein binds left-handed DNA to high levels in the presence of ATP. This complex can be dissociated to some extent with ADP, but most efficiently by single stranded DNA. When dissociation occurs via the latter mechanism, the enzyme becomes associated with the single stranded DNA and is fully capable of participating in homologous pairing reactions. Furthermore, the binding of Z-DNA is stimulated several fold by the presence of B-DNA early in the reaction.

As mentioned, a biological role of left-handed DNA still remains elusive. The firm interaction between Z-DNA and rec1 protein suggests a possible role for Z-DNA in recombination. Since rec1 protein creates

Fig. 8-5. Poisoning of paranemic joint molecules is blocked by Z-DNA. A reaction containing 7.5 μM single-stranded circular M13 DNA, 1 mM ADP, and 15μg/ml rec1 protein, and either 15 μM brominated poly(dG·dC), unbrominated poly (dG·dC), or ϕX linear duplex DNA was incubated at 37°C. After 20 minutes 15 μM ^{3}H-labeled M13 Gori1 linear duplex DNA was added, ATP was regenerated and reaction continued at 37°C as shown. Synaptic complexes when protector DNA was brominated poly(dG·dC) (●); unbrominated poly(dG·dC) (○); linear duplex ϕX DNA (△).


PRESYNAPSIS: single-stranded DNA, ADP, rec I protein, & B-DNA or Z-DNA
SYNAPSIS: Labeled duplexDNA, ATP
paranemic joint
Z-DNA
60
40
20
20
40
60
Time at Synapsis(min)
^{3}H-Labeled DNA Retained (%)

regions of Z-DNA in the formation of a paranemic joint, this DNA binding relationship is not surprising. The results presented in this chapter indicate that polymeric Z-DNA is bound quite tightly, whereas the paranemic joint is easily dissociated. Both DNA-protein complexes are sensitive to dissociation by ADP, which may act by knocking rec1 protein off left-handed helices within the paranemic joint. The Z-DNA binding proteins from Drosophila have been found to bind and stabilize the DNA (Nordheim et al., 1982). The manner in which these proteins were purified, via affinity chromatography, may not identify all proteins capable of binding Z-DNA. Some of them which bind B-DNA as well could have remained bound to the column. Those proteins which were identified, may also function in the interconversion of B- to Z-forms of DNA. The Drosophila genome contains regions of alternating (C-A/G-T)n sequences, but not alternating (G-C) sequences (Hamada and Kalsunaga, 1982). This suggests that Z-DNA binding proteins may have both structural and sequence specifity.

Although the rec1 protein has not been shown to promote a B to Z transition, the stimulation of binding Z-DNA by B-DNA suggests an important relationship between the two DNA forms and the enzyme. Stabilizing stretches of Z-DNA may be a mechanism used by the cell to control gene expression especially on the transcriptional level (Nordheim and Rich, 1983).

CHAPTER NINE
SIGNIFICANCE

The rec1 protein of Ustilago maydis is a novel eukaryotic enzyme. By using ATP as an energy source, this protein catalyzes the formation of a wide range of hybrid DNA molecules. Some of the joint molecules produced in these reactions correlate well with prescribed structural intermediates in the overall recombination pathway. Most significant among these intermediates is the D-loop. Because of the limiting in vitro manipulations afforded by the D-loop reaction, homologous pairing in vitro was studied using single stranded circular and linear duplex DNA. This reaction is appealing for the analysis of homologous pairing because the substrates are easily manipulated and easily assayed. The results of these experiments have demonstrated that the rec1 protein pairs homologous DNA molecules in three distinct phases; presynapsis, synapsis, and strand exchange. Presynapsis involves the preparation of DNA molecules for the pairing reaction. The binding of rec1 protein to single stranded DNA occurs during this phase, and the subsequent interaction with duplex DNA is initiated. The second phase, synapsis, is comprised of several subphases. During the initial stages of synapsis the search for homology brings the DNA molecules into homologous alignment. Then, the conjunction of the two DNA sequences begins. The ternary complex formed during synapsis is comprised of two DNA molecules and rec1 protein held together by DNA-protein interactions but little base-pairing. This nascent heteroduplex is extended by

increasing the length of the heteroduplex in the final phase, strand exchange. Futhermore, each phase and subphase has particular energy requirements while having different susceptibilities to various reaction components. These three phases may define the stages of recombination between two synapsed chromosomes.

Another unique property of the recl protein is its ability to bind tenaciously to left-handed Z-DNA. Although it is not the first Z-DNA binding protein found in eukaryotes, it is the first protein having recombinational activities shown to bind Z-DNA. This observation may implicate Z-DNA as a functional part of the recombination process. The fact that recl protein binds duplex DNA in the Z-form, but not the B-form may also suggest a possible location for recl protein in the cell itself. Since Z-DNA structures exist widespread throughout a number of genomes (see Chapters 5 and 8), this may be a recognition site for initiation of recl protein-promoted strand transfer activities.

These studies have also reinforced the hypothesis that during the initiation of recombination pairing can precede breakage. Results of a number of experiments indicate that recl protein can pair two homologous DNA molecules in the absence of a free end, albeit in an unstable configuration. Subsequent branch migration during strand exchange may eventually incorporate a free end and thus increase the stability of the joint molecule. This mechanism has obvious advantages in the recombination cycle avoiding DNA-damaging nuclease activities on the duplex during the initiation phase. Also problems of topological constraint can be alleviated by the action of topoisomerases. Ustilago

topoisomerase has been shown to act in concert with rec1 protein to carry out such a pairing reaction.

Experimental results from a variety of different reactions have indicated the eminent role ADP plays in controlling rec1 protein pairing activity. ADP destabilizes paranemic joint molecules and promotes dissociation of rec1 protein from all types of DNA, except single stranded. In the presence of single stranded DNA, it inactivates rec1 protein's pairing activities and in this way prevents the synapsis of DNA molecules.

The data presented in this dissertation can be assimilated into a working hypothesis of how the rec1 protein catalyzes strand transfer reactions. This tentative mechanism is depicted in Figure 9-1. The model uses as its prototype a reaction in which rec1 protein is protected from inactivation by nonhomologous duplex DNA and subsequently catalyzes the synapsis of single stranded circular and homologous linear duplex DNA. The first three reaction stages make up the phase of presynapsis while the next two are part of synapsis. The final stage depicted is the beginning of strand exchange.

A) Free rec1 protein binds to single stranded DNA to initiate the presynapsis phase. If a large amount of ADP is prevalent in the reaction environment, the rec1 protein is inactivated and the strand transfer reaction is halted.

B) Nonhomologous DNA duplex DNA is encountered by the complex via a mechanism as yet unknown.

C) A transitory presynaptic complex is complete now with protein molecules spanning the gap between DNA molecules bringing them together. Since optimal protection from ADP inhibition during

presynapsis occurs at a nucleotide ratio of 2:1, duplex to single stranded DNA, it is conceivable that more than one recl protein may be attached to a particular duplex. The enzyme may now change its conformation as it begins tracking along the duplex in search of homology. This movement allows the enzyme to become resistant to inactivation by ADP. This completes the presynapsis phase.

D) The addition of duplex DNA in this particular assay (or a homologous region on the same duplex molecule) to the transitory complex mixture begins the substage of alignment in synapsis. Nonhomologous duplex DNA is maintained at another duplex binding site while the search continues on the homologous synaptic duplex. Alignment occurs and the transition to conjunction begins.

E) This transition involves the condensation of the two DNA molecules via a breathing mechanism of the recl protein. Conformational changes now once again make the enzyme susceptible to ADP inhibition. This movement may also cause the release of the other unaligned complexes which dissociate in various combinations. This is postulated since only one population of product molecules has been observed (RFII) in this particular strand transfer reaction (see Chapter 3).

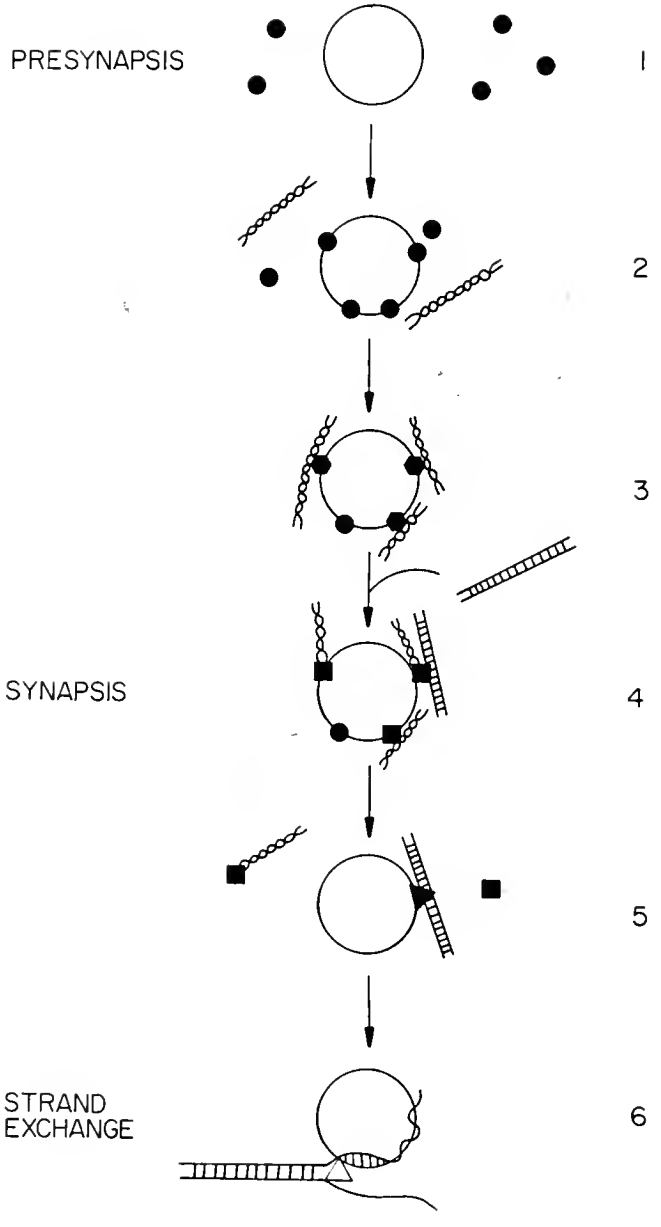
F) Condensation continues leading to initial DNA base pairing between the circular and its complementary strand within the duplex. Once this reannealing begins, recl protein is in a conformation which can unwind the duplex driving the strand exchange using ATP hydrolysis as an energy source and moving in a polar direction. In this position recl protein is resistant to ADP-promoted dissociation, but is sensitive to ADP in another way. ADP inhibits ATP hydrolysis by acting as a competitive inhibitor.

G) If a homologous free end is not available for pairing, dissociation of rec1 protein from duplex DNA can occur via addition of ADP. The torsional strain created by internal pairing results in the formation of left-handed, Z-DNA to which rec1 protein binds. This movement onto the Z-DNA makes the enzyme susceptible to ADP because the binding of Z-DNA is achieved by a final change in conformation.

This model provides a series of mechanistic reaction steps that are currently consistent with the data.

A key feature of this model is the conformational changes which rec1 protein must undergo to interact preferentially with different DNA substrates at different times. Furthermore, some of the changes must take place slowly and reversibly. Hysteretic enzymes are defined as those enzymes which respond slowly to a rapid change in ligand, either substrate or modifer, concentration (Frieden, 1970). Among the conformationally induced enzyme changes are ligand-induced isomerization, displacement of ligands, and polymerization or depolymerization. The enzymes which have been described as hysteretic are often those involved in metabolic regulation (Hatfield and Umbarger, 1970; Gerlt and Rabinowitz, 1970; Frieden, 1970). Enzymes which inhibit such slow transition response could better regulate the cellular responses to metabolic flux. Ainslie et al. (1972) argued that a slow transition of enzyme contributes to their cooperativity, and makes them more competent in their interaction with cellular modifiers. In the case of rec1 protein these modifiers are ATP, ADP and DNA. The complex interactions that occur between these various components and their effect of rec1 protein continues to provide an expanding source of recombination intrigue.

Fig. 9-1. The pairing of homologous DNA molecules promoted by recl protein.


PRESYNAPSIS
1
2
3
SYNAPSIS
4
5
STRAND
EXCHANGE
6

APPENDIX A
BACTERIAL AND FUNGAL STRAINS

Ustilago maydis strains 58, 56, 293, 221 and d58, d56 were obtained from Dr. Robin Holliday, National Institute for Medical Research, London. The cells were cultivated in medium consisting of 2% peptone, 2% sucrose, 1% yeast extract in a Virtis fermenter with vigorous aeration. Bacteriophage ϕX174 am3 and its host E. coli HF 4704 and phage M13 Gori1 were acquired from Dr. Charles Radding, Yale University. Bacteriophages fd and M13 and their host E. coli K37 were acquired from Dr. W. Konigsberg. Bacteriophage P22 and its host Salmonella typhmurium DB25 were acquired from Dr. David Botstein. E. coli SA 920 (λ int29, cI 857 X13) was obtained from Dr. Max Gottesman, National Institute of Health. AB 2463 (λ recX314 cI 857) were obtained from Dr. R.P. Boyce, University of Florida.

APPENDIX B
PREPARATION OF DNA STOCKS

P22 DNA and ϕX174 DNA were prepared as described by Carter and Radding (1971). fd phage, and fd RFI DNA were prepared as described by Cunningham et al. (1980). G4 and M13 DNA was prepared as described by Beattie et al. (1977). DNA from the hybrid phage M13 Gori1 (Kaguni and Ray, 1979) was prepared as described for M13. All concentrations of DNA were expressed as moles of nucleotide residue unless otherwise indicated. Specific activities of all DNAs used in this study ranged from 1 to 4 x 10^4 cpm/µmole.

APPENDIX C
PREPARATION OF DNA SUBSTRATES

P22 [^{3}H-DNA was heat denatured by boiling for 5 mintues followed by quick cooling in ice. ϕX RFI DNA was converted into the nicked RFII by treatment with Pancreatic DNase (Stettler et al., 1979). Single-strand circular DNA from ϕX174 and fd phages was fragmented by heating at 100°C for 15 minutes.

Restriction endonuclease digestion of M13 Gori1, M13, fd, ϕX, G4 RFI DNA was carried out as described in Kmiec and Holloman (1983; or Kmiec et al., 1983). Labeling of 3' or 5' ends of linear duplex DNA and purification of specific fragments was carried out as described in Kmiec and Holloman (1983). D-loop DNA was prepared by thermal annealing as described in Beattie et al. (1977), purification of D-loop DNA was carried out as described in Kmiec et al. (1983). Renaturation of DNA after sedimentation in alkaline sucrose was carried out as described in Lau and Gray (1980).

Poly(dG·dC) was purchased from P-L Biochemicals and brominated by the method described by Lafer et al. (1981). Conversion of brominated poly(dG·dC) to the left-handed form was monitored using a Jasco J500C spectropolarimeter to follow inversion of the circular dichroism spectrum. Poly(dG·dC) was labeled with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase (Weiss et al., 1968). Efficiency of labeling brominated and unbrominated poly(dG·dC) was similar. Specific activity of each was approximately 1 x 10^{4}

cpm/nmol. All DNA concentrations are expressed as moles of nucleotide. Preparative amounts of specific restriction fragments were purified by electrophoresis in low melting agarose and the DNA reisolated by melting appropriate slices from the gel as described by Maniatis et al. (1982).

APPENDIX D
PROTEIN PURIFICATION

Purification of the recl protein was acheived as described in Kmiec and Holloman (1982). For these studies, fraction V was used. This fraction contained 6 μg/ml protein in 10 ml, DNA-dependent ATPase activity at 150,000 units/mg and ATP-dependent reannealing activity at 65,000 units/mg. To study the level of enzyme activity in the mutant strains, fraction IV with an ATPase activity of 37,300 units/mg was used.

β protein was purified as described in Radding et al. (1971) with a slight modification as described in Kmiec and Holloman (1981).

Topoisomerase was purified as described by Rowe et al. (1981). The specific activity was approximately 10^6 units/mg and the purity was greater than 50% (unpublished observations).

APPENDIX E
ASSAYS

Reannealing

Reaction mixtures of 50 µl contained 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothrietol, 0.5 mg/ml bovine serum albumin, 1.0 mM ATP, 2 nmol denatured P22 [^{3}H]DNA (2.5 x 10^4 cpm/nmol) and appropriate amounts of the protein. After incubation at 37° for 45 minutes 0.45 ml of 0.2 M sodium acetate pH 4.5, containing 2 mM $ZnCl_2$ was added together with enough S_1 nuclease (Bethesda Research Laboratories) to completely hydrolyze 10 nmol of denatured P22 DNA and incubation was continued for 30 minutes. After addition of carrier DNA and trichloroacetic acid the radioactivity remaining soluble was determined. One unit of reannealing activity is defined as that amount of protein causing 1 nmol DNA to become resistant to S_1 hydrolysis under the conditions described.

D-loop Formation

Reaction mixtures of 100 µl contained 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothrietol, 0.5 mg/ml bovine serum albumin, 1.0 mM ATP, 7.5 µM RF1[^{3}H]DNA, 8.0 µM single stranded fragments and Ustilago pairing protein. After incubation at 37° for 45 minutes 100 µl 25 mM EDTA were added followed by 1 ml of 1.5 M NaCl, 0.15 sodium citrate (10x SSC). The mixture was washed onto a nitrocellulose filter (Schleicher and Schuell type BA85), processed as described by Beattie

et al. (1977), and the radioactivity remaining bound to the filter was determined.

Strand Transfer

Reaction mixtures of 100 µl were prepared as in the D-loop assay except that linear duplex RFIII DNA was substituted for RFI DNA and intact single strand circular DNA was substituted for fragments. After incubation, mixtures were filtered through nitrocellulose and processed as in the D-loop assay.

ATPase

Reaction mixtures of 15 µl contained 20 mM Tris·HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothrietol, 0.5 mg/ml bovine serum albumin, 1.0 mM [^{3}H] ATP (3700 cpm/nmol), 1 nmol X174 DNA and the Ustilago protein. After incubation at 37° for 45 minutes, excess EDTA and unlabeled ATP, ADP, and AMP were added as visual markers, and the entire mixture spotted onto a sheet of PEI-cellulose (EM Laboratories) and chromatographed in 1M formic acid, 0.5 M LiCl. Spots containing the marker nucleotides were located under ultraviolet light, cut out, and the radioactivity determined. One unit of ATPase activity is defined as the amount of protein that hydrolyzes ATP to form 1 nmol of ADP under the conditions described.

Joint Molecule Formation

The method of assaying joint molecules by retention on nitrocellulose filters was described before (Kmiec and Holloman, 1982) as the D-loop assay. Reaction mixtures contained 50 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothrietol, and DNA and rec1 protein as described in the figure legends. After incubation at 37° EDTA was added to a final concentration of 10 mM and reaction mixtures

were diluted 25-fold with cold 1.5 M NaCl, 0.15 M sodium citrate, pH 7.5 containing 0.16% SDS. Mixtures were then passed through nitrocellulose filters (Schleicher and Schuell, BA85), processed as described by Beattie et al. (1977) and the radioactvity remaining bound was determined.

Joint molecule formation was also determined with use of an assay that measured the extent of the heteroduplex region. Reaction mixtures of 40 µl as described above contained ^{3}H-labeled circular single stranded DNA and unlabeled linear duplex DNA. After incubation at 37° with recl protein, reaction mixtures were diluted with 460 µl S-1 cocktail (0.25 M potassium acetate, pH 4.5, 2 mM $ZnCl_2$) and enough S-1 nuclease to digest completely five times the amount of single stranded DNA present. After an additional 30 minutes incubation, 250 µg carrier DNA was added followed by trichloroacetatic acid to a final concentration of 5%. After 10 minutes on ice, mixtures were passed through glass fiber filters (Whatman, GF/C), the filters rinsed twice with 0.5 ml of 5% trichloroacetate acid, twice with 0.5 ml of ethanol, dried, and the bound radioactivity determined. The increase in acid precipitable radioactivity upon addition of recl protein to reactions was taken as a measure of heteroduplex formation.

Protein-Binding Assay

Standard pairing reactions contained 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM ATP, 7.5 µM single-stranded circular DNA, 15 µM ^{3}H-labeled linear duplex DNA and 15 µg/ml recl protein.

Ternary complexes were measured by monitoring retention of ^{3}H-labeled DNA on nitrocellulose filters. EDTA was added to reactions to 25 mM to stop reaction and prevent any further binding

then reaction mixtures were immediately filtered through nitrocellulose filters at a rate of 1 ml/minute. Filters were washed twice with 2 ml portions of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, dried under a heat lamp, and the bound radioactivity was determined by scintillation counting in toluene base fluor. Nitrocellulose filters (Gelman Metricel GN-6 membranes) were prepared by heating in a boiling solution of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA for 10 minutes immediately before use. Efficiency of the assay was monitored using, as a control, H1 histone, which binds to single-stranded and double-stranded DNA equally well (Palter and Alberts, 1979). When bound by H1 histone, a maximum of 85 to 90% of input single-stranded or duplex DNA was reproducibly retained by filters from batch to batch. In the absence of added protein, 4 to 8% of labeled single-stranded DNA was retained by filters and 2 to 4% of duplex DNA was retained. Nitrocellulose filters from Sartorious, Schleicher and Schuell, or Millipore were much less satisfactory in this assay.

Formation of left-handed DNA was detected with the use of Z-DNA antibody. Wells of Cooke microtiter plates were coated with Z-DNA antibody by filling each with 100 µl of a solution containing 20 µg/ml goat Z-DNA antibody (IgG fraction) in 20 mM potassium phosphate, pH 7.5, and allowing plates to stand at 22°C for 24 hours. Unabsorbed antibody was removed, wells were flooded with 20 mM potassium phosphate, pH 7.5, containing 0.3 M NaCl, and 100 µg/ml bovine serum albumin. After 24 hours at 22°C plates were blotted dried. To assay for left-handed DNA, solutions containing ^{3}H-labeled linear duplex DNA were brought to 50 mM EDTA and 50 µl was added to a well. After 2 hours at 22°C wells were washed three times by flooding with 20 mM

potassium phosphate, pH 7.5, 0.3 M NaCl, 0.001% Tween-20, 100 µg/ml bovine serum albumin. Plates were dried, the wells cut out, placed in scintillation vials and radioactivity derermined. When up to 4 nmol ^{32}P-labeled brominated poly(dG·dC) was bound per well, less than 0.25 nmol ^{32}P-labeled unbrominated poly(dG·dC) was bound.

APPENDIX F
METHODS AND MATERIALS

Enzymes and Reagents

Exonuclease I from E. coli B, purified through hydroxylapatite, according to Lehman and Nussbaum (1964), was prepared by M. Yarnall of this laboratory. Yeast hexokinase was purchased from Sigma Chemical Co. S-1 nuclease, restriction endonucleases BamHI, XhoI, HpaI, and Sau96I, as well as large fragment DNA polymerase I were all from Bethesda Research Laboratories. Adenylyl-imidodiphosphate was from Boehringer Mannheim. Proteinase K was from E. Merck. Creatine phosphate and creatine phosphokinase were from Sigma Chemical Co. One unit of creatine phosphate is the amount required to convert 1 nmole of ATP to ADP per minute at 37°C. Z-DNA antibody from goat was the kind gift from Professor David Stollar, Tufts University. RNA polymerase was E. coli holoenzyme from Sigma Chemical Co. The activity was measured by the method of Burgess and Jendrisak (1975) in reactions containing 35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM [^{3}H]ATP (7.3×10^4 cpm/nmole) 0.15 mM CTP, 0.15 mM, GTP, 0.15 mM UTP, 80 μM fd from I DNA, and RNA polymerase. One unit is that amount of activity that incorporates 1 nmole of ATP into an acid-insoluble product in 10 minutes at 37°C.

Sucrose Gradient Centrifugation

Centrifugation of DNA under alkaline conditions was carried out in 5%-20% sucrose solutions containing 0.3 M NaOH, 0.7 M NaCl, and 1 mM

EDTA. Fractions collected at the end of a run were reneutralized with an equivalent amount of HCl and the radioactivity was determined by scintillation counting using Triton Fluor. When necessary, DNA from alkaline gradients was renatured according to the procedure of Lau and Gray (1980). Ethidium bromide was added to the alkaline fractions containing DNA to a final concentration of 160 µg/ml, and the solution was kept at 25°C for 12 hours. The DNA was then dialyzed against 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1 mM EDTA, freed of ethidium bromide by passage through a small Dowex-50 column, and concentrated by precipitation with ethanol.

Centrifugation under neutral conditions was carried out in 5%-20% sucrose gradients containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and either 1 M NaCl or 0.01 M NaCl, where indicated. The direction of sedimentation in all gradients is from right to left.

Gel Electrophoresis

DNA was analyzed in 1.2% agarose gels containing 40 mM Tris acetate, pH 7.9, 5 mM sodium acetate, 1 mM EDTA. Electrophoresis was carried out at room temperature with 2 volts/cm for 18 hours. Gels were stained with ethidium bromide at 1 µg/ml and photographed under ultraviolet light with Polaroid type 55 film.

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BIOGRAPHICAL SKETCH

Eric Kmiec was born and raised in Ware, Massachusetts. After attending Ware High School, he went on to Rutgers College where he majored in microbiology, receiving his B.A. in 1978. From 1978 to 1980, he studied cell biology at Southern Illinois where he received his M.S. degree in June, 1980. More importantly, he met Jennifer Lynn Bartz in one of his embryology classes and married her 18 months later. They moved to Gainesville where Eric earned his Ph.D. in 1984.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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